EXPERIMENTAL SHIGELLOSIS IN MICE

I. CHRONIC INFECTION WITH *SHIGELLA DYSENTERIAE* TYPE 2

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SUMMARY.

A chronic infection of *Sh. dysenteriae* 2 can be produced in mice following oral administration of approximately one hundred organisms suspended in broth. Prior treatment of the animals with Streptomycin and Erythromycin is necessary to establish the infection and continued antibiotic treatment prevents the re-establishment of the normal bowel flora. Macroscopic and microscopic evidence of pathological damage, consistent with the findings in natural *Shigellosis* in man, has been described. It is claimed that the mouse infection resembles a chronic form of bacillary dysentery and therefore serves as a suitable experimental system for the investigation of many basic problems connected with the pathogenesis of, and immunological response to, *Shigella* infection.

INTRODUCTION.

In 1953 Burrows and Ware stated that "other than with cholera, few if any of the enteric diseases of man may be reproduced in experimental animals in truly enteric form". Since then, however, several groups of workers have developed techniques which have apparently successfully produced experimental intestinal infections with *Salmonella* and *Shigella* species.

Bohnhoff, Drake and Miller (1954) indicated that the susceptibility of mice to oral infections of *Salmonella enteritidis* was greatly increased if it was preceded by a large oral dose of Streptomycin. About the same time, Freter (1955) described the use of orally administered Streptomycin to potentiate a fatal infection of *Vibrio cholerae* in guinea-pigs, given by the oral route. Later the same worker (Freter, 1956) showed that infection of mice with *Sh. flexneri* was also possible using a similar technique. While the latter infection was not fatal, the animals were shown to excrete the infecting organisms in large numbers for some time; it was presumed, without histological evidence, that an intestinal infection had been set up in the animals.

The present communication describes in greater detail the features of this oral infection of *Shigella* in mice. The original technique described by Freter

has been modified to some extent, providing a simple means of establishing an intestinal colonization of the organism which, it is claimed, simulates the chronic *Shigella* infection of man.

Materials and Methods.

Animals. Adult mice weighing between 20 gm. and 25 gm. were used throughout this work. It was found that mice derived from a number of different strains were equally susceptible to the infection. The mice were kept in individual cages throughout an experiment; the cages were designed so that the excreta fell through a wire mesh and accumulated in sterile Petri dish covers which were placed beneath the cages. The wire mesh was sufficiently small to retain the solid cubes of "dog meal" given to the animals for food.

Organisms. The strain of *Sh. dysenteriae* 2 (Prismall) had been isolated from a patient in October, 1956, and maintained in the dry state. Streptomycin-resistant mutants (S^r) of this strain were isolated by inoculating lawn cultures on nutrient agar containing 100 µg./ml. Streptomycin. The S^r variants which developed were recovered and maintained in the dry state. These strains were found to be biochemically and serologically identical with the parent strain. When required, the organisms were recovered from the dry state in Hartley's tryptic broth medium (H.T.B.).

Mode of infection. Using a poly-vinyl chloride stomach tube, animals were given 0·2 ml. of a solution containing 5 mg. Streptomycin and 1 mg. Erythromycin. They were placed in their individual cages and given, *ad lib.*, food and boiled tap water containing 0·5 mg. Streptomycin and 0·01 mg. Erythromycin per ml. Two days later the animals were given, by stomach tube, 0·2 ml. of a suspension containing 50 mg. $CaCO_3$ and 5 mg. Streptomycin. A second stomach tube was then passed and a known inoculum of *Sh. dysenteriae* 2 (S^r) suspended in 0·2 ml. of H.T.B. was given to each animal. The mice were then returned to their cages and given food and Streptomycin-Erythromycin water, *ad lib.*, throughout the course of the experiment.

Faecal cultures. The faeces of the infected animals were collected at daily intervals from the Petri dish covers beneath the cages. To compare the extent of excretion by each animal the following technique was adopted: twenty faecal pellets, weighing approximately 1 gm. were transferred by sterile forceps to 5 ml. of 0·85 p.c. saline in 1 oz. screw-capped bottles. The material was shaken mechanically for one hour to prepare a relatively homogeneous faecal suspension.

A 4 mm. loop-full of the suspension was then streaked on to an Eosin-Methylene Blue medium containing 1 p.c. mannitol and 1 p.c. lactose. The plates were incubated at 37° C. for 24 hours and *Sh. dysenteriae* 2 developed as 1 mm. circular, smooth, colourless colonies. The organism was thus differentiated from the lactose or mannitol fermenting species of enteric bacteria which also may have been present. Confirmation of the identity of the organisms was carried out by biochemical reactions and slide agglutination with specific antisera.

RESULTS.

In an initial experiment a group of ten mice were infected by the above technique with a dose of approximately 1×10^4 *Sh. dysenteriae* 2 (S^r) organisms. A second group of ten mice were given the same inoculum of organisms but Streptomycin and Erythromycin were withheld from them throughout the course of the experiment. A third group, treated with antibiotics, received a similar dose of Streptomycin-sensitive *Sh. dysenteriae* 2. Faecal cultures were made daily after infection and the results are recorded in Table 1.

TABLE 1.

Mouse infection with Shigella dysenteriae 2 *strains.*

Mice given	No. of animals challenged	No. of animals excreting *Sh. dysenteriae* 2
1. *Sh. dysenteriae* 2 (S^r) +Antibiotics	10	10
2. *Sh. dysenteriae* 2 (S^r) without Antibiotics	10	0
3. *Sh. dysenteriae* 2 (S^s) +Antibiotics	10	0

It will be noted that faecal excretion of *Sh. dysenteriae* 2 occurred only in those mice which were treated with antibiotics and given the resistant strain of the organism. In these animals the organisms were isolated in large numbers from the faeces within 24 hours of infection; excretion continued for a period of three weeks, at which stage the experiment was terminated.

Determination of infecting doses. Three groups of ten mice were given graded doses of the *Sh. dysenteriae* 2 (S^r) strain by the usual technique. The infecting inocula were derived from the eighteen-hour growth on Hartley's tryptic agar and suspended in Hartley's tryptic broth; serial tenfold dilutions of this suspension were prepared in Hartley's tryptic broth and the inocula selected from three of these dilutions. The number of organisms given to the animals in each group was determined by viable counts. 0·2 ml. volumes of the inocula were delivered on to a quadruplicate series of Hartley's tryptic agar plates and spread with an angled-glass spreader. After 24 hours' incubation at 37° C. the colonies were counted, and the average number obtained on the four plates was regarded as the infecting dose of organisms for the particular group of mice.

As before, faecal cultures from all animals were prepared at daily intervals after infection. The results of three similar experiments are recorded in Table 2. It is obvious that under the experimental conditions imposed here, the inoculum required to set up an infection with *Sh. dysenteriae* 2 is quite small.

TABLE 2.

Infection of mice with Shigella dysenteriae 2 (S^r).

EXPERIMENT 1		EXPERIMENT 2		EXPERIMENT 3	
No. of organisms given orally	Infection rate	No. of organisms given orally	Infection rate	No. of organisms given orally	Infection rate
1,665	10/10	1,385	10/10	2,290	10/10
153	10/10	127	10/10	188	10/10
18	3/10	15	2/10	27	4/10

It has been possible to produce infection regularly with about one hundred organisms and, in some instances, as few as fifteen to twenty organisms have successfully initiated faecal excretion.

In certain instances the animals were kept for periods of twenty-eight days after infection; *Sh. dysenteriae* 2 could be isolated from the faeces throughout this period. It was found, however, that introduction of Streptomycin-resistant strains of *Escherichia coli*, either accidentally or purposely, caused the rapid elimination of *Sh. dysenteriae* 2 from the faeces, thus confirming the observations of Freter (1956).

Macroscopic evidence of infection. Following infection it was noted that the mice produced substantially greater amounts of faeces than normal animals: moreover, the faecal pellets were much larger and softer than normal. Microscopic examination of the faeces did not reveal the presence of blood or mucus, however, nor could occult blood be detected by the Benzidene test. The animals remained clinically well throughout the course of the infection and no deaths have been recorded in many hundreds of animals subjected to the experimental infection.

At various intervals after infection the mice were killed by coal gas and their peritoneal cavities exposed. Progressive changes in the macroscopic appearance of the intestinal tract of the infected animals were found during the first fourteen days of the infection, but little alteration occurred thereafter. The characteristic features found at necropsy in infected mice were as follows:

(i) The intestines were congested and dark grey in colour, in contrast to the pale yellow of normal mouse intestines.

(ii) The appendix was markedly longer than normal, grossly distended with gas and characteristically doughy to the touch. It was dark brown in colour and showed some congestion and haemorrhage.

(iii) The caecum was congested, haemorrhagic and grossly distended with gas.

(iv) All other organs of the mice appeared normal.

Uninfected mice which had been given the same antibiotic treatment as the infected animals were examined at the same time as infected animals. While the intestinal tract was found to be normal in colour and not congested, it was found that the appendix of these animals was longer than in normal animals. In contrast with the infected animals, however, this organ was not distended with gas. The caecum was enlarged but was not congested or haemorrhagic.

Sh. dysenteriae 2 was always isolated from the appendix, caecum and colon of the infected mice. Less frequently was the organism recovered from the upper intestinal tract, while on rare occasions it was found in the gastric contents; the organism was never isolated from the liver, spleen, kidneys or heart blood of infected mice. It is claimed, therefore, that the infection is localized in the intestinal tract of the animals.

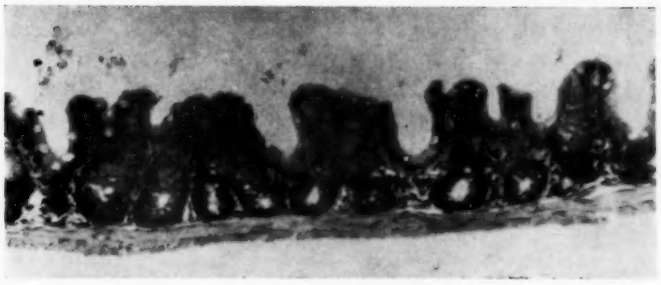

Fig. 1. *Section of normal mouse appendix.* Haematoxylin-Eosin Stain (x 70).

Fig. 2. *Section of appendix of mouse infected eighteen days previously with Sh. dysenteriae* 2. Haematoxylin-Eosin Stain (x 70).

Histological evidence of infection. Sections of various regions of the intestinal tract of normal, infected and antibiotic-treated-uninfected animals were prepared for histological examination. While it was a general feature in the infected mice that some degeneration of the mucosa of the upper intestinal tract occurred, the most characteristic changes were found in the appendix and caecum. Gross mucosal destruction was found in these sites, while in many areas a marked infiltration of inflammatory cells, extending into the sub-mucosa, was also noted. The muscularis was not apparently affected. Figs. 1, 2 and 3 compare the histological findings of the appendix of normal, infected and Streptomycin-Erythromycin-treated-uninfected mice. Histologically, the latter animal resembles the normal mouse and it is apparent that the increased size of the appendix of these animals is not associated with any pathological damage.

Histological examination of animals throughout the course of the infection indicated that the pathological changes were first obvious within five to seven

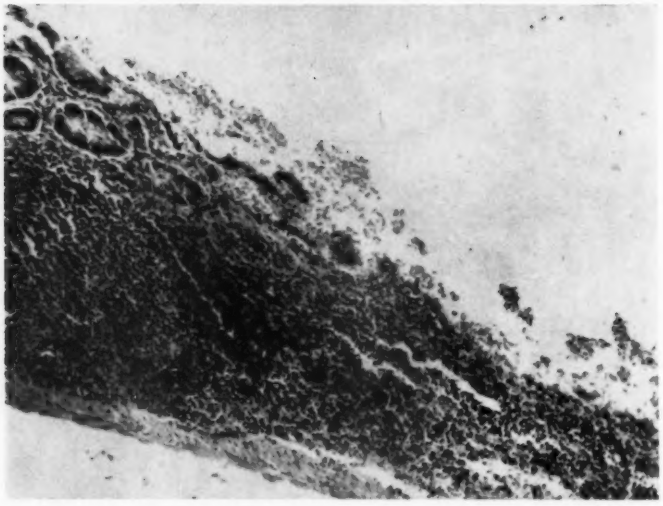

Fig. 3. *Section of appendix of non-infected mouse given Streptomycin and Erythromycin for eighteen days.* Haematoxylin-Eosin Stain (x 70).

days. Degeneration of the mucosa and an accumulation of lymphocytes in the sub-mucosa occurred initially. Later the mucosa was completely destroyed in the appendix, associated with an inflammatory cell infiltration. No further changes were found after fourteen to eighteen days of the infection and in several animals examined about the twenty-eighth day some decrease in inflammatory response and regeneration of the mucosa was observed.

DISCUSSION.

The Freter technique has been adapted to produce a non-fatal, chronic infection with *Sh. dysenteriae* 2 in mice. It has been shown that, following the oral administration of small infecting inocula, the mice excrete large numbers of the organism in their faeces within twenty-four hours, and may continue to do so for long periods thereafter. While Freter (1956) has shown that substantially the same occurs following infection with *Sh. flexneri* in mice, it was felt his claim that infection had been established, based only on the faecal excretion of the organisms, was not necessarily justified. It was not possible on such evidence to distinguish between a true enteric infection and what may be termed an "intestinal colonization".

In the present communication, however, it has been shown that marked pathological changes do occur in mice. Gross distension and congestion of the

appendix and caecum regularly occur following infection, and histological examination has shown this to be associated with inflammatory cell infiltration and mucosal destruction. The histological findings resemble those described in the pathology of bacillary dysentery in man (MacCallum, 1940). While the characteristic lesions in the mouse are localized in the appendico-caecal areas and ulcer formation does not regularly occur, sufficient similarity exists to justify the claim that the murine infection may be closely allied to the natural infection. As such, it provides an admirable experimental system for subsequent examination of host responses to *Shigella* infection.

The mode of action of the antibiotics in potentiating this infection is of particular interest. It could readily be shown that the normal bacterial flora of the intestinal tract was rapidly eliminated following the oral antibiotic treatment; moreover, *Shigella* infection could not be set up until this was accomplished. Presumably mechanisms which have broadly been termed as "bacterial antagonisms" operate to prevent multiplication of the *Shigella* in normal mice. In addition, the infection may readily be eliminated following the introduction of non-pathogenic, Streptomycin-resistant *Esch. coli*. Freter's investigations on these aspects have not provided a clue to these mechanisms, save that they certainly are not due to "colicin" or antibiotic production.

Recently Formal *et al.* (1958) have described a fatal infection with orally-administered *Sh. flexneri* 2a in guinea-pigs. The histological findings in these infections are very similar to those recorded here. In contrast to the Freter technique, however, these infections were set up with large inocula given after a four-day fasting period. At the time of infection a large amount of calcium carbonate was given with the suspension of organisms, and tincture of opium was injected intraperitoneally one hour after the administration of the organisms. The effect of starvation is probably different from that of the antibiotics used in Freter's technique; there is no evidence to suggest that the former method either eliminates or reduces the normal bacterial flora. Although Freter's original technique involved the fasting of the animals for two days prior to infection, the evidence presented here indicates it is not an essential step in the potentiation of the infection.

While the antibiotic effects are undoubtedly related to the removal of the bacterial flora in the intestinal tract, it may be that other factors are also involved. While no histological change in the intestines of the antibiotic-treated animals was found, the increased length of the appendix of the animals suggests that some changes have occurred which may allow the establishment of the *Shigella* in this site and its subsequent rapid multiplication. Tentatively it may be suggested that this change is related to reduced permeability of the mucosa, preventing the accumulation of certain non-specific protective factors which may normally inhibit the multiplication of the potentially pathogenic organisms.

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EXPERIMENTAL SHIGELLOSIS IN MICE

II. IMMUNOLOGICAL RESPONSES TO *SHIGELLA DYSENTERIAE* TYPE 2 INFECTION

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SUMMARY.

The immunological response of mice to *Sh. dysenteriae* 2 has been investigated. Whereas serum agglutinins did not appear regularly until the second week, and reached a maximal level about the fourteenth day, antibodies were excreted in the faeces earlier in the infection. Generally the maximal copro-antibody response occurred on the seventh day and was not detected after fourteen days; considerable irregularity in copro-antibody production by individual mice was noted. Evidence has been presented which indicates that a certain proportion of the lymphocytes derived from infected mice contain specific *Sh. dysenteriae* 2 antibodies. These were detected originally by the lymphocyte adherence phenomena and subsequently by the haemagglutination technique. As yet the rôle of the lymphocytes in *Shigella* infection has not been established.

INTRODUCTION.

Studies in the pathogenesis of, and resistance to, bacillary dysentery have been limited owing to the inability to produce infections in laboratory animals which simulate the natural disease of man. Although Fairbrother and Hirst (1932), Dack and Petran (1934), and Preston and Clark (1938) had suggested that *Shigella* infections produced in monkeys, either naturally or experimentally, resembled the human infection, later workers, including Rewell and Bridges (1948) and Branham *et al.* (1949), indicated that such infections occur irregularly and may represent a toxaemia rather than the localized bowel infection characteristic of the natural disease in man. However, in the previous communication it has been established that, following the oral administration of small numbers of *Sh. dysenteriae* 2, a localized intestinal infection regularly develops in mice, producing pathological changes which bear a striking similarity to those of the human infection. The present report describes certain of the immunological responses of the mice to this infection and, as will be seen, provides support for the belief that the natural human and experimental murine infections are similar.

MATERIALS AND METHODS.

Infection in mice. Mice weighing 20 to 25 gm. were infected by the oral route with approximately 100 to 150 organisms suspended in Hartley's tryptic broth (H.T.B.), using the technique described previously. Cultures from the faeces of all infected mice were made at daily intervals to ensure that excretion of the organisms continued throughout the course of the experiments. At the completion of an experiment all animals were sacrificed and their peritoneal cavities were exposed and examined for macroscopic evidence of infection.

Faecal extracts for copro-antibody determinations. Twenty faecal pellets weighing approximately one gm. were collected from each animal and placed in 5 ml. of 0·5 p.c. phenol saline. The faeces were shaken mechanically for one hour and the homogeneous suspension was centrifuged at 2,000 r.p.m. for 20 minutes. The supernatant fluid was recovered and heated at 56° C. for 30 minutes to destroy complement.

Serum preparation. At specified periods after infection the animals were sacrificed to collect serum for agglutinin investigations. The mice were exsanguinated under anaesthesia by severing the axillary blood vessels (Cooper, 1952). The blood was collected in 8 cm. × 0·8 cm. tubes, allowed to clot at room temperature and the serum withdrawn after centrifugation of the blood clot. The sera were always absorbed with 10 p.c. sheep red cells and heated to 56° C. for 30 minutes before use.

Haemagglutination. Antibody levels in sera and faecal extracts were determined by the indirect bacterial haemagglutination technique. Five per cent. washed sheep cells were sensitized with an equal volume of *Sh. dysenteriae* 2 haemosensitin dissolved in saline. The haemosensitin was prepared by alkaline hydrolysis of the organism and subsequently purified by repeated acetone fractionation; sensitizing solutions contained 50 μg. of haemosensitin per ml. This concentration was found to be five times the concentration required to sensitize sheep cells so that they produced a maximum haemagglutination titre with a standard *Sh. dysenteriae* 2 antiserum. Haemosensitization (Boyden and Suter, 1952) was carried out at 37° C. for two hours. The sensitized cells were then centrifuged, washed three times in large volumes of physiological saline and finally resuspended in saline to a concentration of 0·5 p.c.

Haemagglutination reactions were carried out in perspex trays which contained moulded cups. The sera or faecal extracts were serially diluted in 0·1 ml. volumes using the method of Takatsy (1955) and 0·1 ml. of sensitized cells was added to each dilution. The tests were inspected after one hour's incubation at 37° C. and 30 minutes at room temperature. The antibody titre was regarded as the reciprocal of the highest dilution of serum or faecal extract causing complete agglutination of the sensitized cells.

RESULTS.

Serum antibody levels following infection.

Sixty mice were infected with *Sh. dysenteriae* 2 by the oral route. Within 24 hours all animals were found to be excreting large numbers of the organism in their faeces, and continued to do so throughout the course of the experiment. On the 4th, 7th, 10th, 14th, 21st and 28th days of infection groups of ten animals were bled under anaesthesia, the individual sera being tested for the presence of *Sh. dysenteriae* 2 antibodies. Initial serum dilutions used in the haemagglutination reactions were 1 in 5. The serum haemagglutination titres of each mouse used in this experiment are recorded in Table 1, together with the average serum titre found at each of the days following infection. While some mouse to mouse

TABLE 1.

Serum antibody levels of mice following Shigella Dysenteriae 2 *infection.*

Days after infection	Haemagglutination titres (Serum No.)										Average titre
	1	2	3	4	5	6	7	8	9	10	
4	< 5	5	20	< 5	10	5	10	< 5	< 5	10	6
7	80	20	40	40	20	< 5	10	40	40	10	30
10	80	160	40	80	40	80	80	160	80	40	84
14	160	80	320	320	160	160	80	80	160	80	160
21	40	160	80	80	20	5	80	40	160	10	67·5
28	10	5	20	40	20	20	10	5	10	40	18

variation has occurred, the results indicate the general trend of the serum antibody response to infection. During the first week of infection the antibody level is quite low, but it rapidly increases to a maximal level about the fourteenth day of the infection. Although the antibody level decreases considerably during the following two weeks, it is nonetheless apparent that all animals retain some level of circulating antibody at this stage of the infection.

Copro-antibody levels following infection.

It was shown initially by Davies (1922) that during a dysentery infection in man, specific antibodies were excreted in the faeces. Since then a number of workers, including Predpenchensky *et al.* (1940) and Yampolsky *et al.* (1944), have confirmed their appearance in the early stages of *Shigella* infection. It was of interest, therefore, to estimate the faecal excretion of antibody during the experimental mouse infection.

Forty mice were infected with approximately 100 *Sh. dysenteriae* 2 organisms by the oral route. Cultures at daily intervals indicated that all animals continued to excrete the organism throughout the course of the experiment. On each of the first 14 days after infection then on the 21st and 28th days, faecal suspensions from each mouse were prepared in phenol saline and the haemag-

TABLE 2.

Copro-antibody production during Shigella Dysenteriae 2 *infection in mice.*

	Days after infection															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	21	28
Average copro-antibody titre	0	0·9	1·8	2·4	2·8	2·5	3·7	3·8	2·4	2·3	2·1	0·9	1·3	0·7	0	0
Percentage animals excreting copro-antibody	0	p.c. 33	p.c. 58	p.c. 60	p.c. 63	p.c. 54	p.c. 97	p.c. 91	p.c. 75	p.c. 66	p.c. 60	p.c. 30	p.c. 38	p.c. 26	0	0

glutination titre of the extracts determined. In all instances initial dilutions of the faecal extracts were 1 in 2. Average copro-antibody titres for the forty animals on each day after infection are recorded in Table 2 together with the daily percentage of animals found to be excreting the antibody.

The results suggest that small amounts of copro-antibody are excreted shortly after the infection has been set up. Maximal excretion appears to occur about the seventh or eighth day of the infection and a rapid decrease in copro-antibody production occurs thereafter. This is supported also by the percentage of animals excreting detectable amounts of antibody on each day of the infection. If copro-antibody production is compared with serum antibody levels throughout the course of the infection (Fig. 1), it is evident that the latter response to infection is far more regular and of longer duration than that of the copro-antibodies.

Examination of the copro-antibody production by individual mice throughout the infection further suggested its irregularity. The mouse to mouse varia-

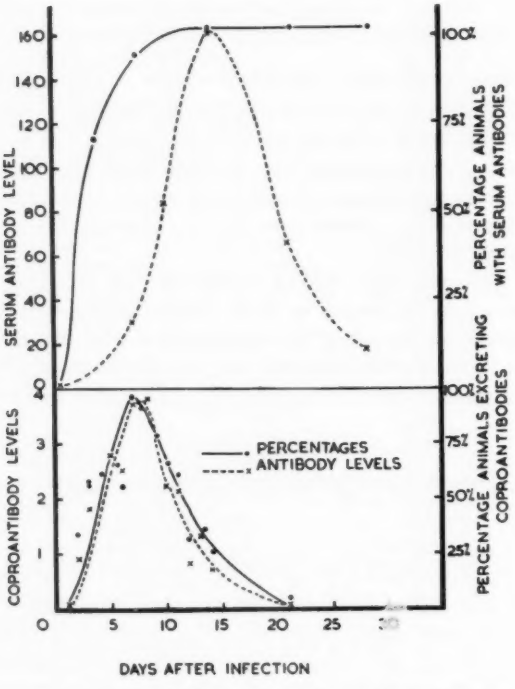

Fig. 1. Copro-antibody and serum antibody levels in mice following *Shigella dysenteriae* 2 infection.

tion was considerable and, while the maximal response of all animals appeared at the seventh or eight days, considerable fluctuation occurred in duration of the response and the levels obtained. To a certain extent this may have been influenced by variation inherent in the technique that was adopted. Nonetheless, in many instances mice were found to excrete detectable amounts of antibody for only two or three days, while in some cases antibodies were found for periods of nine or ten days. On occasions mice were found to produce the copro-antibodies at irregular intervals, although in all instances the highest level of copro-antibody was detected on the seventh or eighth days of the infection.

Cellular activity in infected mice.

Following the oral infection of mice with *Sh. dysenteriae* 2 an accumulation of lymphocytes and polymorphonuclear cells occurs in the sub-mucosa of the appendix and caecum of the animals. A similar cellular response to *Shigella* infection is observed in the natural disease; it therefore seemed important to investigate any possible *in vitro* activity of these cells against the infecting organism.

Groups of four mice were infected with *Sh. dysenteriae* 2 in the usual manner. Seven days later each animal was given 2 ml. of sterile 10 p.c. peptone in saline by the intraperitoneal route. After 14 hours the animals were killed by coal gas and the peritoneal cavities washed out with 10 ml. of physiological saline. The peritoneal washings of the four mice were pooled, centrifuged and washed five times in saline and finally resuspended in 5 ml. of saline. Smears of the suspensions, stained by Leishman's method, showed them to contain approximately 60 p.c. lymphocytes, 35 p.c. polymorphonuclear cells and 5 p.c. monocytes, and direct counts in a haemocytometer were of the order of 1,500 cells per cmm. One ml. volume of the cell suspensions was mixed with an equal volume of a saline suspension of living *Sh. dysenteriae* 2 containing 50×10^6 organisms per ml. The mixtures were incubated at 37° C. for 30 minutes, then centrifuged at 2,000 r.p.m. for 20 minutes. The deposits were smeared and stained by Leishman's method.

Although the polymorphonuclear cells did not show any phagocytosis, such a result was not surprising in view of the absence of serum from the experimental system. However, it was observed with approximately 10 to 15 p.c. of the lymphocytes that a number of organisms (usually 8 to 12 in number) were apparently physically adhering to their surface. Similar adherence phenomena have been observed with immature plasma cells (Reiss *et al.*, 1950; Nossal, personal communication) and lymphocytes (Hayes and Dougherty, 1954) with entirely different experimental systems. In these instances the phenomenon was related to antibody production by the particular cells. Further investigations of this phenomenon were therefore undertaken.

Groups of four mice were again infected with *Sh. dysenteriae* 2 and groups of four normal animals were set aside as controls. Seven days later both infected

and normal animals were injected subcutaneously on the back with 0·5 ml. volumes of a water-in-oil emulsion containing 2 mg. per ml. dried and killed *Mycobacterium tuberculosis* (a Freund-type adjuvant). Fourteen days later all animals were killed by coal gas. At necropsy, considerable enlargement of the mesenteric lymph nodes and of those of the lumbar lymphatic chain was found. The lymph nodes were dissected out and those from each group of four animals were placed in a small volume of sterile Ringer solution. The lymph nodes were "teased" out with a pair of fine needles and the resulting suspensions were passed through a sterile, fine, stainless steel mesh to remove all coarse particles. The suspensions were centrifuged and washed five times in Ringer solution and the washed cells were finally suspended in 10 ml. volumes of saline. Smears showed the suspensions to consist almost entirely of small, round, basophilic cells which were assumed to be small lymphocytes; the suspensions contained approximately 3,000 cells per cmm.

Lymphocytes from normal and infected animals were mixed in equal volumes (one ml.) with saline suspensions of living *Sh. dysenteriae* 2 cells. As controls the lymphocyte suspensions were mixed with similar suspensions of *Sh. flexneri* 2a, *Esch. coli, Proteus vulgaris, Pseudomonas pyocyanea* and *Staph. pyogenes.* Following incubation at 37° C. for 30 minutes cell suspensions were centrifuged and the deposits smeared and stained by Leishman's method. Twelve to 15 p.c. of the lymphocytes in suspensions from infected groups of animals were found to exhibit the "lymphocyte-adherence" phenomenon with *Sh. dysenteriae* 2 cells. No similar phenomena were observed with normal lymphocytes nor with those from the infected animals in the presence of the heterologous bacterial suspensions.

The lymphocyte suspensions from both infected and normal groups of animals were examined for their ability to produce haemagglutination of the sensitized sheep cells. Twofold dilutions of the lymphocyte suspensions were prepared in 0·1 ml. volumes in the perspex trays and 0·1 ml. volumes of 0·5 p.c. sheep red cells, sensitized with *Sh. dysenteriae* 2 haemosensitin, were added. As controls, sheep cells sensitized with the haemosensitins of *Esch. coli, Sh. sonnei, Sh. flexneri* 2a and *Salm. typhi-murium* were added to similar dilutions of the lymphocytes. The results recorded after incubation of the tests as described previously are shown in Table 3. The results demonstrate conclusively the participation of *Sh. dysenteriae* 2 antibodies in the reaction. Further substantiation of this was obtained from the following type of experiment.

One ml. volumes of lymphocyte suspensions were centrifuged and the deposited cells were resuspended in one ml. of distilled water. Rapid lysis occurred and the cell stromata were removed by centrifugation at 4,000 r.p.m. for 30 minutes. The cell lysates were collected and the stromata were resuspended in one ml. of saline. The cell lysates were mixed with an equal volume of 1·7 p.c. saline and, as before, serial twofold dilutions were prepared in 0·1

TABLE 3.

Haemagglutination by lymphocytes.

Sheep cells sensitized with haemosensitins of	INFECTED MICE Lymphocyte dilutions						NORMAL MICE Lymphocyte dilutions					
	N	1/2	1/4	1/8	1/16	1/32	N	1/2	1/4	1/8	1/16	1/32
Sh. dysenteriae 2	+	+	+	+	−	−	−	−	−	−	−	−
Sh. sonnei	−	−	−	−	−	−	−	−	−	−	−	−
Sh. flexneri 2a	−	−	−	−	−	−	−	−	−	−	−	−
Salm. typhi-murium	−	−	−	−	−	−	−	−	−	−	−	−
Esch. coli	−	−	−	−	−	−	−	−	−	−	−	−
Approximate No. of lymphocytes per cmm.	3,000	1,500	750	375	188	94	3,000	1,500	750	375	188	94

ml. volumes for use in the haemagglutination reaction. It was found that cell lysates from infected animals caused agglutination of sheep cells sensitized with *Sh. dysenteriae* 2 up to a dilution of 1 in 8. This approximated to the extract from 188 cells per cmm. As before, it was found that normal cell lysates did not cause haemagglutination, while the lysates from the infected animals failed to agglutinate sheep cells sensitized with heterologous haemosensitins. No haemagglutination activity of the cell stromata could be detected.

The results have indicated that a proportion of the lymphocytes obtained from animals infected with *Sh. dysenteriae* 2 contain specific antibody. Several experiments designed to test whether these antibodies were lethal or inhibitory for *Sh. dysenteriae* 2, either in the presence or absence of complement, have been undertaken. The results have not indicated any such activity and it must be concluded, on the evidence so far available, that these antibodies are similar to the normal serum antibodies.

DISCUSSION.

Harrison and Banvard (1947) have shown in a number of *Shigella*-infected patients that in the initial stages copro-antibodies are excreted and reach a maximal level about the eighth or ninth day of the infection. On the other hand, serum antibodies were rarely detected before the seventh day of the disease; generally the serum antibody level attained its maximal level between the 14th and 21st days and remained at a relatively high level for some period thereafter. The results recorded here, using a mouse infection with *Sh. dysenteriae* 2, are very similar. While the copro-antibody response occurred early in the infection, it was found that the serum antibodies develop later and may be maintained for some period after infection, although not at such high levels as in the human infection. The similarity of the immunological responses of man

and mouse to the *Shigella* infection cannot be overlooked and probably adds further weight to the claim that these infections may indeed be very similar.

From an immunological and pathological view, Asiatic cholera and Shigellosis may be considered to be very similar in that both are infections which are localized in the intestinal tract. Moreover, in neither disease are the mechanisms of host resistance well understood. It is well appreciated, however, that serum antibody levels in individuals bear little relationship to the immune state, probably by virtue of their inaccessibility to the mucosa of the intestinal tract. Burrows and Ware (1953) using *Vibrio cholerae* infections in guinea-pigs and Freter (1956) using *Sh. flexneri* in mice have shown, however, that oral administration of high-titred homologous antisera may protect animals from these experimental infections, although it was unable to modify the course of the disease, once established. Copro-antibody production during *Shigella* infection has been observed by many workers (*vide supra*) although Gonzalez *et al.* (1950) were unable to detect their presence in the faeces of a number of patients infected with *Sh. flexneri.* Similarly, Burrows and Havens (1948) have detected *V. cholerae* antibodies in faeces of humans and experimentally infected guinea-pigs. Again, Koshland and Burrows (1950) have shown that copro-antibody production occurs in guinea-pigs immunized with *V. cholerae* vaccine. These observations have led to the suggestion that copro-antibodies may be an essential factor in protection against these infections. Burrows and Havens have further tentatively suggested that they may be released from lymphocytic cells which accumulate at or near the site of infection.

While an overall increase in copro-antibody production has been observed during the first seven days of the experimental mouse infection, individual mouse to mouse variations have been of such an order as to suggest that the excretion of these antibodies is not necessarily a regular feature of the infection. This finding may explain, in part, the inability of Gonzalez *et al.* (1950) to detect copro-antibodies following *Sh. flexneri* infection. Moreover, considerable variation in the amount of detectable antibody excreted by the animals over any particular period was noted. In view of these irregularities it is difficult at this stage to accept the belief that these antibodies represent an especial defence mechanism against the localized bowel infection.

The results recorded here may support Burrows' hypothesis of the lymphocytic origin of the faecal antibodies. Certainly it has been shown that a proportion of lymphocytes derived from *Shigella*-infected mice possess specific antibodies which may be detected by relatively coarse *in vitro* techniques. Moreover, the increased haemagglutination titre of the cell lysates suggests that the antibodies are contained within the cell. The results, however, do not either support or deny that the antibodies are produced by the lymphocytes. It is possible, as Wissler *et al.* (1957) have recently suggested, that they may be derived from an antibody-producing cell and act as carriers of the antibody to

the site of infection. It is not possible at this stage to estimate the significance of the lymphocytic antibodies in the protection of the host against the *Shigella* infection; further investigation of their protective nature is undoubtedly warranted, however.

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THE RESPONSE TO Na^+ DEPLETION IN ADRENALECTOMIZED SHEEP WITH PAROTID FISTULAE[1]

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SUMMARY.

Bilaterally adrenalectomized sheep with a parotid fistula were maintained in good condition indefinitely if they received 5 mg. DOCA and 25 mg. cortisone/day, and adequate $NaHCO_3$ to replace that lost from the parotid fistula.

Na^+ depletion produced by adrenal insufficiency did not cause a fall in the parotid salivary Na^+/K^+ ratio.

If Na^+ depletion were produced by withholding the daily Na^+ supplement, and the sheep received only basal maintenance dosage of DOCA (5 mg./day), the Na^+ deficit caused little fall of salivary Na^+/K^+ ratio. Signs of adrenal insufficiency developed in 2-4 days.

If, during Na^+ deficit, the adrenalectomized sheep were given gradually increasing dosage of DOCA reaching six times basal, the parotid response was similar to that of a normal sheep during Na^+ depletion.

If adrenalectomized sheep were given an increased but constant DOCA dosage for 8-10 days, an equilibrium state with a considerably lowered salivary Na^+/K^+ ratio resulted, and if the daily Na^+ supplement were withdrawn then, the salivary Na^+/K^+ ratio decreased to the level seen during Na^+ depletion in a normal sheep. Restoration of the Na^+ supplement caused the ratio to return to that pertaining for the high DOCA dosage. This experiment, which eliminated the possibility of variable adrenal steroid action, showed that one or more factors in addition to the adrenal cortex were involved in the parotid response to Na^+ deficit.

The relation between salivary secretion rate and Na^+/K^+ ratio was examined in the above experiments. Also experiments were made in which the decreased secretion rate during Na^+ deficit was reversed by intracarotid ACh infusion. These experiments showed that a local parotid factor associated with decreased secretion rate was mainly responsible for the decrease of ratio during Na^+ deficit when increased but constant DOCA was given. The results indicated that *there are only two major factors in the parotid response to Na^+ deficit in a normal sheep.* These are an increased secretion of electrolyte-active adrenal steroid, presumably mainly aldosterone, and a local parotid factor associated with decreased secretion rate. There was also evidence that the sensitivity of the parotid gland to electrolyte-active steroid increased during Na^+ depletion.

Consecutive collections of saliva, in Na^+-depleted adrenalectomized sheep, gave evidence of the quantitative effect and duration of a single dose of DOCA.

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The applicability of the terms "permissive" and "contributory cause" as applied to the action of the adrenal hormones in electrolyte regulation is discussed.

INTRODUCTION.

If Na^+ depletion occurs in a sheep with a permanent parotid fistula the salivary Na^+/K^+ ratio falls. Deficits of 500-1000 m-equiv. of Na^+ cause the salivary Na^+/K^+ to decrease from a normal value of $180/10 = 18$ to as low as $10/180 = 0{\cdot}06$ (Denton, 1956, 1957a). Experiments aimed at defining the mechanism of this parotid response have shown:—

(i) Na^+ depletion in a normal sheep was associated with a large decrease in secretion rate. If the rate were returned to normal by intracarotid infusion of acetylcholine (ACh) the salivary Na^+/K^+ ratio rose, but only to the extent of 20-50 p.c. of the deviation from normal. Therefore, during Na^+ depletion in the normal sheep, a factor associated with decrease in secretion rate was involved in the resultant fall in salivary Na^+/K^+ ratio. However, it was clear that the major factor had to be sought elsewhere (Denton, 1957b).

(ii) If Na^+ depletion were produced by withdrawal of the daily adrenal hormone injections from a bilaterally adrenalectomized sheep, the usual parotid response did not occur, though urinary loss caused a Na^+ deficit of as much as 310 m-equiv. (Goding and Denton, 1956, 1957).

(iii) The intramuscular injection of desoxycorticosterone acetate (DOCA) caused a large fall of salivary Na^+/K^+ ratio in both normal and adrenalectomized sheep (Goding and Denton, 1956, 1957). This fall was much greater than that observed in the Na^+/K^+ ratio of human mixed saliva following the injection of DOCA (Frawley and Thorn, 1951) and aldosterone (Mach, Fabre, Duckert, Borth and Ducommun, 1954).

(iv) The rapid removal of both adrenal glands from an anaesthetized Na^+ depleted sheep, with a low salivary Na^+/K^+ ratio, was followed by a rapid rise of the ratio after a time delay of 120 min. (Denton and McDonald, 1957).

(v) Drainage of the entire adrenal venous affluent from conscious undisturbed Na^+ deficient sheep with transplanted adrenal glands had a similar effect on the salivary Na^+/K^+ ratio as (iv) (McDonald, Goding and Wright, 1958).

These results indicated that the electrolyte-active secretion of the adrenal cortex was essential for the normal parotid response to Na^+ depletion, and injection of electrolyte-active adrenal steroids produced an effect on the salivary Na^+/K^+ ratio similar to Na^+ depletion.

This paper reports experiments in which Na^+ depletion was produced in adrenalectomized sheep with a parotid fistula by withdrawal of the daily Na^+ supplement. The parotid response was studied under various conditions of hormone dosage.

It was found that, if the sheep were depleted of Na^+ whilst receiving only the usual maintenance dose of DOCA (5 mg./day), the Na^+ deficit caused little fall of salivary Na^+/K^+ ratio. After 2-4 days the evident signs of adrenal

insufficiency necessitated replacement of the daily Na^+ supplement. If, however, the adrenalectomized sheep were given gradually increasing amounts of DOCA during the period of Na^+ deficiency, the salivary response was similar to that seen during Na^+ depletion in the normal animal. A series of experiments was carried out in which the sheep were given constant, but greatly increased, daily DOCA supplement (20 or 40 mg./day) for some days before, during and after the period of Na^+ depletion. This amount of DOCA caused a gradual fall in salivary Na^+/K^+ ratio over an initial period of 3-4 days. The ratio then remained at this lower level without further fall. If the daily Na^+ supplement were withdrawn then, the ratio fell commensurately with the Na^+ deficit to the level seen during Na^+ depletion in an intact animal. Upon restoration of Na^+ intake it rose again to the equilibrium level pertaining to the high DOCA dosage.

These results indicated that an increased amount of electrolyte-active steroid was necessary for the normal parotid response to Na^+ deficit. The experiments on increased but constant DOCA dosage, insofar as they eliminated the possibility of variation of adrenal steroid action, showed that one or more factors in addition to the adrenal cortex were involved in the parotid response to Na^+ deficit in the normal animal.

As indicated in (i), diminished parotid secretory activity played a part in the normal response to Na^+ depletion. In order to determine the extent to which this factor was the cause of the response obtained in the adrenalectomized sheep, two types of experiment were carried out. Firstly, the relationship between the daily salivary volume and the Na^+/K^+ ratio was studied. Secondly, experiments were made in which the decreased salivary secretion rate caused by Na^+ depletion was returned to normal for short periods on succeeding days of depletion by intracarotid infusion of ACh. The results suggested that the decrease of salivary Na^+/K^+ during Na^+ depletion in the bilaterally adrenalectomized sheep maintained on increased but constant dosage of DOCA was largely a consequence of the decreased secretory activity. Hence it is inferred that there are only two major components in the mechanism of parotid response to Na^+ depletion in the normal animal, viz., an increased secretion of electrolyte-active hormone by the adrenal gland and a local parotid factor associated with decrease of secretion rate.

Methods.

Eight crossbred Merino sheep were used. All had permanent unilateral parotid fistulae prepared by the Wright technique (Denton, 1957a), and four, Adr. 1, P.F.20, Adr. 8 and Adr. 11, were bilaterally adrenalectomized in the manner described by Goding and Denton (1957). All except Adr. 1 had carotid loops (van Leersum, 1911). Sheep Adr. 11 had a unilateral nephrectomy.

The adrenalectomized sheep remained in good condition indefinitely if given daily intramuscular injections of 5 mg. DOCA (Percorten, Ciba Ltd., Basle) and 25 mg. cortisone (Cortistab, Boots), and a daily intrarumenal supplement of 300-417 m-equiv. (20-35 gm.) $NaHCO_3$.

Diet. The sheep were fed throughout on their normal diet of a mixture of oaten and lucerne chaff. This provided a daily intake for Adr. 1 of 70 m-equiv. Na^+ and 318 m-equiv. K^+; for P.F.20, 23 and 234 m-equiv.; and for Adr. 8 and Adr. 11, 172 and 242 m-equiv.

The procedure for maintenance of adrenalectomized animals, the conduct of total electrolyte balance studies in stainless steel metabolism cages, the technique of intracarotid infusion of ACh and 4 M NaCl, and the chemical methods have been described elsewhere (Denton, 1956, 1957a, b; Goding and Denton, 1957; Denton and McDonald, 1957).

RESULTS.

The results of the experiments reported below have been described in two sections. The first and principal section deals with the changes in parotid saliva during Na^+ depletion, and the second section gives details of the corresponding relevant changes in other physiological functions.

SECTION 1.

The effect of various conditions of hormone dosage on the changes in salivary Na^+/K^+ ratio of adrenalectomized sheep during Na^+ depletion and repletion.

The results showed that adrenal steroids must be provided in amounts greater than basal maintenance dosage if Na^+ depletion were to result in the usual fall in salivary Na^+/K^+ ratio. In subsection (a), Na^+ depletion was caused by increased urinary loss of Na^+ following hormone withdrawal; in sections (b)-(d) Na^+ depletion was caused by continued parotid secretion while the Na^+ supplement was withheld; in (e) both causes were operative.

(a) *Withdrawal of hormone supplement, Na^+ supplement continued.* Three experiments were performed, two on Adr. 1 and one on P.F.20. In these withdrawal of DOCA alone, or of both DOCA and cortisone, caused Na^+ deficits of 136-310 m-equiv., even though the daily Na^+ supplement was continued. This was due to greatly increased urinary loss of Na^+. In each experiment the salivary Na^+/K^+ ratio rose, and there was a decrease in the daily volume of saliva to ½ to ⅓ of the control volume. Full balance studies have been presented elsewhere (Goding and Denton, 1957).

A typical experiment is shown in Fig. 1. Withdrawal of DOCA for two days from adrenalectomized sheep P.F.20 resulted in a Na^+ deficit of 310 m-equiv.; however, there was no fall in salivary Na^+/K^+ ratio – in fact, it rose (▲ ---- ▲). In a normal sheep, P.F.17, a Na^+ deficit of this order, caused by withholding Na^+ supplement, resulted in a significant fall in salivary Na^+/K^+ ratio (○ ---- ○).

Hence in the absence of adrenal hormones, the normal salivary response to Na^+ depletion, did not occur.

(b) *Maintenance level of hormone supplement, Na^+ supplement withdrawn.* In five experiments, using sheep Adr. 1 (3 experiments), P.F.20 and Adr. 8, the sheep were maintained on daily injections of 5 mg. DOCA and 25 mg. cortisone.

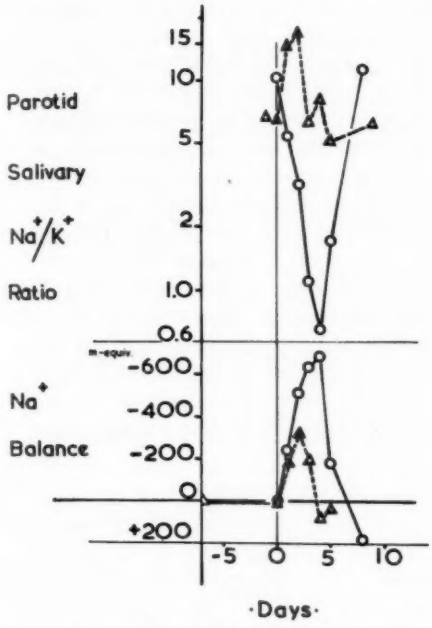

Fig. 1. *The relationship between salivary Na+/K+ ratio and Na+ balance following withdrawal and replacement of DOCA dosage in an adrenalectomized sheep, P.F.20.* For comparison there is an episode of Na+ depletion in a normal sheep with a parotid fistula, P.F.17. △—P.F.20, 10 mg. DOCA/day. ▲—P.F.20, no DOCA. ○—P.F.17.

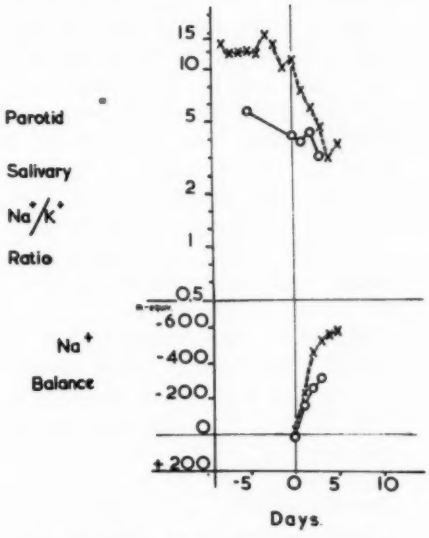

Fig. 2. *The relationship between salivary Na+/K+ ratio and Na+ balance when the $NaHCO_3$ supplement was withdrawn from adrenalectomized sheep on maintenance dosage of hormones.* (Cortisone, 25 mg./day, DOCA 5 mg./day). ×—Adr. 8; ○—P.F.20. $NaHCO_3$ withdrawn on Day 0.

They were given their usual diet. Na+ depletion was caused by withholding the Na+ supplement for 2-5 days. The results of two such experiments are shown in Fig. 2.

In sheep P.F.20 (○ ---- ○), after three days' Na+ depletion there was little change in the salivary Na+/K+ ratio despite a Na+ deficit of 311 m-equiv. By the third day the salivary secretion rate had fallen to one-quarter of the control rate.

In sheep Adr. 8 (× ---- ×), after 5 days' depletion, the salivary Na+/K+ ratio fell from 11·2 to 3·7, with a Na+ deficit of 574 m-equiv. This fall is much less than would be shown by a normal sheep (Fig. 1). The salivary secretion rate had fallen to one-eighth of the average control rate by the end of the Na+ depletion period. The experiments on sheep Adr. 1 gave the same result.

Hence, in the presence of maintenance level of hormone supplement only the salivary response to Na+ depletion was considerably less than normal.

(c) *Graded increase of hormone supplement during a period of withdrawal of Na^+ supplement.* This experiment was performed once on sheep Adr. 1 (Fig. 3, × ---- ×). In the seven-day control period, the sheep was given its normal diet, a Na^+ supplement of 300 m-equiv. $NaHCO_3$ and a hormone supplement of 5 mg. DOCA and 25 mg. cortisone per day. The average salivary Na^+/K^+ ratio in the control period was 10·8, and the average daily volume of saliva was 1·93 l. The $NaHCO_3$ supplement was withdrawn then for 7 days, and during this period the hormone supplement was increased according to the following plan – first day, cortisone 75 mg., DOCA 15 mg.; second day, 100, 20; third day, 150, 30; fourth to seventh day, 100, 20. A Na^+ deficit of 439 m-equiv. resulted. The salivary Na^+/K^+ fell progressively from 10·8 to 0·89 (Fig. 3, × ---- ×) in the same way as that of a non-adrenalectomized sheep during deprivation of its $NaHCO_3$ supplement. The rate of salivary secretion did not decrease much during the first 5 days, but fell over the last 2 days to 0·5 l./day. As control to this experiment, the daily hormone supplement of Adr. 1 was increased in a similar way, without withdrawing the $NaHCO_3$ supplement. This caused a considerable fall in salivary Na^+/K^+, but of much less degree (Fig. 3, ○ ---- ○). The secretion rate increased by 50 p.c. In two other experiments on P.F.21 (a non-adrenalectomized sheep) and Adr. 8, a step-wise increase of cortisone alone produced no significant change in the salivary Na^+/K^+.

Hence with a concurrent step-wise increase of electrolyte-active hormone supplement, the normal salivary response to Na^+ depletion occurs.

(d) *Hormone supplement increased but constant before, during and after Na^+ withdrawal.* It has been shown (Goding and Denton, 1957) that a fourfold increase in the daily DOCA supplement given to an adrenalectomized sheep in normal Na^+ balance caused the salivary Na^+/K^+ ratio to fall. However, this ratio then became stable at a level which was always higher than that found in gross Na^+ depletion in normal sheep. Accordingly, it was necessary to determine whether Na^+

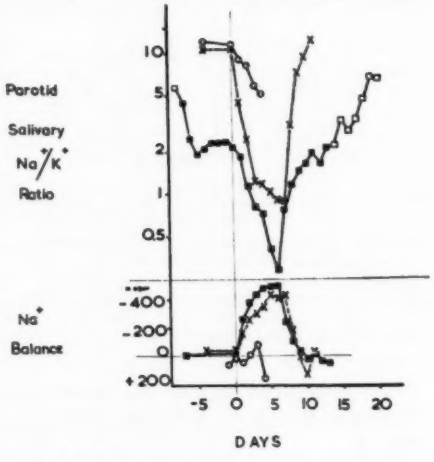

Fig. 3. *The relationship between salivary Na^+/K^+ and Na^+ balance when the $NaHCO_3$ supplement was withdrawn from adrenalectomized sheep.* ×—Adr. 1, Na^+ withdrawal covered by graded increase in hormone dosage (see text). ○—Adr. 1, control experiment in which hormone dosage was increased without Na^+ withdrawal (see text). P.F.20, Na^+ withdrawal while on constant but increased DOCA dosage. □ — DOCA 10 mg./day. ■ — DOCA 20 mg./day (Na^+ withdrawn on Day 0).

depletion under conditions of increased but constant daily DOCA supplement would cause the ratio to fall further. Five such experiments were made, three on P.F.20 and one each on Adr. 1 and Adr. 8. They were each carried out in five stages. The cortisone supplement (25 mg./day) was constant throughout all experiments. A typical experiment on P.F.20 is described below (Fig. 3, □-■-■-□).

Stage A. Control period: 10 *mg. DOCA*, 300 *m-equiv. $NaHCO_3$ daily* (3 *days*). The average salivary Na^+/K^+ ratio was 5·1 and the average salivary volume was 1·86 l./day.

Stage B. DOCA supplement increased to 20 *mg./day*, 300 *m-equiv. $NaHCO_3$ daily* (8 *days*). The salivary Na^+/K^+ ratio fell progressively during the first three days to 1·9, and remained in the range of 2·1-2·4 for the next five days. The average saliva volume rose to 2·37 l./day, in keeping with the previous findings of Goding and Denton (1957) on the effect of high DOCA dosage. Although the Na^+ concentration of the saliva fell during this period, the increase in salivary volume was such that the Na^+ supplement was inadequate to replace the salivary loss, and a small Na^+ deficit (76 m-equiv.) resulted.

Stage C. DOCA still 20 *mg./day, Na^+ supplement withdrawn* (6 *days*). The salivary Na^+/K^+ ratio and the secretion rate both fell progressively. On the last day of Na^+ depletion, the Na^+/K^+ ratio was 0·3 and the saliva volume was 0·16 l. There was a Na^+ deficit at the end of this period of 515 m-equiv.

Stage D. DOCA still 20 *mg./day, Na^+ supplement replaced*, 300 *m-equiv. daily* (7 *days*). The salivary Na^+/K^+ ratio and the secretion rate both rose progressively during the first 5 days to the same level as Stage B. During the last 3 days of the period the average Na^+/K^+ ratio was 1·9 (1·7-2·1) and the average saliva volume was 2·39 (2·06-2·86) l./day.

Stage E. Control period: 10 *mg. DOCA*, 300 *m-equiv. $NaHCO_3$ daily* (7 *days*). The salivary Na^+/K^+ ratio rose gradually and was 6·6 and 6·4 on days 6 and 7. The average saliva volume was 2·11 (1·40-2·46) litres.

The results of the other four experiments were similar. During Stage C, Na^+ deficits of 342-925 m-equiv. resulted in salivary Na^+/K^+ ratios of 1·2-0·5. In each case Na^+ depletion was associated with a progressive fall in the rate of salivary secretion.

These experiments showed that increased but constant dosage of DOCA lowered the salivary Na^+/K^+ ratio, and that an equilibrium state was reached. Upon withdrawal of the Na^+ supplement, Na^+ deficits of 342-925 m-equiv. occurred, and the salivary Na^+/K^+ ratio decreased to the levels found during Na^+ depletion in a normal sheep. The salivary Na^+/K^+ ratio returned to the increased DOCA equilibrium ratio upon restoration of the Na^+ balance.

(e) *Hormone supplement and Na^+ intake varied independently.* This experiment illustrates three further aspects of the results described in section (d). Firstly, the sheep P.F.20 was given a considerably larger $NaHCO_3$ supplement (417 m-equiv. daily) during Stages B and C, and hence there was no Na^+

deficit when the DOCA supplement was increased. This, incidentally, enabled further observations to be made on the interdependence of the effects of alterations in Na^+ supplement and DOCA dosage on the salivary Na^+/K^+ ratio. Secondly, an episode (Stage E) is described in which both DOCA and cortisone were withheld for one day when the sheep had a Na^+ deficit of 715 m-equiv. Thirdly, during Stage F, relatively rapid changes were made in the Na^+ supplement while the DOCA supplement was held constant at 20 mg./day. The resultant changes in salivary Na^+/K^+ were observed. The results described below are shown in Fig. 4. Cortisone (25 mg.) was given each day except on the one day referred to as Stage E.

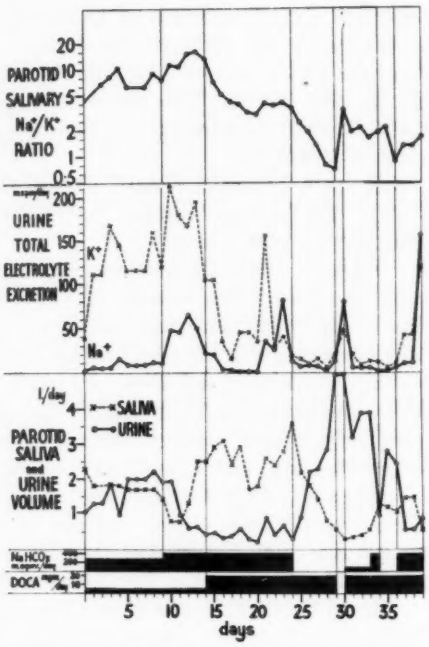

Fig. 4. *The effect on parotid salivary Na^+/K^+, total daily urinary excretion of Na^+ and Ka^+, and volume of saliva and urine, when the Na^+ intake and hormone dosage were varied* (P.F.20).

Stage A. Control period: DOCA 5 mg. $NaHCO_3$ 300 m-equiv./day (9 *days*). The average salivary Na^+/K^+ ratio was 7·8 (6·4-10·7) and the average saliva volume 1·74 (1·48-1·90) l./day.

Stage B. DOCA 5 mg. daily, Na^+ supplement increased to 417 m-equiv./day (5 *days*). The salivary Na^+/K^+ ratio rose to a maximum of 16·2 by the 3rd day, and was 13·4 on the 5th day. There was an initial fall in salivary volume to 0·8 l./day and a subsequent rise to 2·6 l./day: the average for the period was 1·6 l./day.

Thus under these conditions the salivary Na^+/K^+ ratio increased when secretion rate fell and vice versa. This is usually the case over 24-hour collection periods in normal sheep when they are loaded with Na^+.

Stage C. DOCA increased to 20 mg. $NaHCO_3$ still 417 m-equiv./day (10 *days*). The salivary Na^+/K^+ ratio fell gradually during the first 5 days to 3·3, and remained in the range 3·2-4·2 thereafter. In the same sheep, with the same DOCA supplement but only 300 m-equiv. $NaHCO_3$ daily, the salivary Na^+/K^+ ratio stabilized in the range 2·1-2·4. There was the usual sustained rise in saliva volume: the average for the period was 2·65 (1·76-3·54) litres.

Stage D. DOCA 20 mg/day, Na^+ supplement withdrawn (5 *days*). There was the usual progressive fall in salivary Na^+/K^+ ratio and secretion rate. On

the last day of the period the salivary Na^+/K^+ ratio was 0·74, and the saliva volume was 0·57 l. The Na^+ deficit was 715 m-equiv.

Stage E. Na^+ supplement, DOCA and cortisone all withdrawn (1 *day*). The salivary Na^+/K^+ ratio rose abruptly to 3·4 while the volume of saliva secreted fell to 0·21 l.

Stage F. DOCA supplement replaced (20 *mg./day*), *$NaHCO_3$ intake varied* (9 *days*). During this phase the daily $NaHCO_3$ supplement was varied from 0-417 m-equiv. As shown in Fig. 4, the salivary Na^+/K^+ ratio followed the rise and fall in Na^+ intake. Because of the combined effects of Na^+ depletion and adrenal insufficiency in Stages D and E, the salivary volume remained low during Stage F. Hence the loss of Na^+ from the fistula was greatly reduced, and the large $NaHCO_3$ supplement given in the last 3 days caused a net Na^+ gain of 804 m-equiv. The animal died suddenly at the end of Stage F in a state of alkalosis and hypokalaemia (Goding and Denton, 1957).

This experiment, like those reported in section (d), showed that, if an adrenalectomized sheep were given a constant high DOCA supplement, the Na^+/K^+ ratio of the saliva rose and fell in direct relationship with the Na^+ intake. This relationship was lost when the adrenal hormones were withdrawn. The latter result has been confirmed repeatedly. For example, in an experiment on sheep Adr. 11, the salivary Na^+/K^+ ratio was in the range 10·0-15·0 when the sheep was given 5 mg. DOCA, 25 mg. cortisone, Na^+ intake 300 m-equiv./day. A Na^+ deficit of 710 m-equiv., caused by withholding the Na^+ supplement while on 25 mg. cortisone and 20 mg. DOCA daily, resulted in a salivary Na^+/K^+ ratio of 0·45. Serial saliva collections were made commencing 22 hrs. after the previous DOCA injection (Fig. 5). The salivary Na^+/K^+

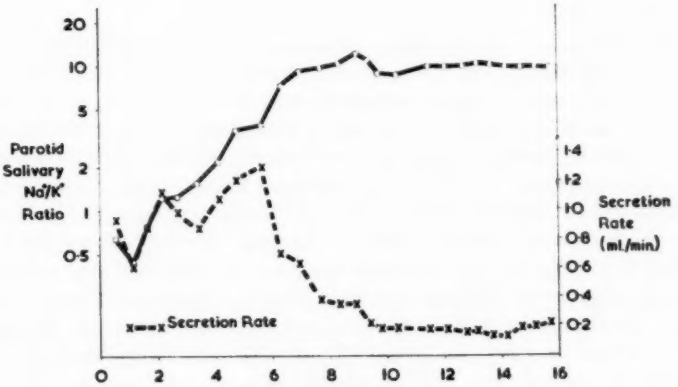

Fig. 5. *The effect of DOCA withdrawal on salivary volume and Na^+/K^+ ratio during Na^+ depletion.* Collections commenced 22 hr. after previous dose of DOCA (20 mg.). Adr. 11.

ratio rose gradually over the ensuing 6 hrs. to 9·1 even though the salivary secretion rate decreased to 0·2-0·4 ml./min. There was little variation thereafter, the ratio remaining in the range 8·6-12·1 for the next 6 hrs.

Hence, during Na^+ depletion the salivary Na^+/K^+ ratio returned to normal by 24-30 hrs. after the previous injection of DOCA. Thus estimation of the Na^+/K^+ ratio of serial collections of saliva may be used to indicate the end-point of action of a previously injected dose of DOCA. In these circumstances the result is clear-cut, as variations in secretion rate are without effect.

Summary of results of Sections 1 (*a*) *to* (*e*). It has been shown that electrolyte-active adrenal steroids do alter the sheep's salivary Na^+/K^+ ratio, and it is a necessary condition that they be administered in excess of basal maintenance dose for the ratio to decrease in the normal way during Na^+ depletion. The unequivocal parotid response to Na^+ deficit when the adrenalectomized animal received increased but constant DOCA dosage suggested that the cause of the parotid behaviour in the normal animal was the simultaneous action of at least two factors. The secretion of increased amounts of electrolyte-active adrenal steroids appeared to be a contributory condition in a set of jointly sufficient and severally necessary conditions for the response to occur.

The effect of altered parotid secretion rate on salivary Na^+/K^+ ratio of adrenalectomized sheep, during Na^+ depletion and repletion.

In the preceding sections it was shown that the parotid salivary Na^+/K^+ ratio fell when Na^+ was withdrawn from adrenalectomized sheep on constant high DOCA dosage. As changes in parotid secretion rate have been shown to affect the salivary Na^+/K^+ ratio, investigations were made on the correlation between changes in salivary secretion rate and Na^+/K^+ ratio when the effect of DOCA was constant.

In non-adrenalectomized sheep, changes associated with altered parotid secretory activity during Na^+ depletion account for 25-40 p.c. of the fall in salivary Na^+/K^+ ratio. The diminished secretory activity may be due to a number of factors, e.g., reduced plasma volume and blood pressure, causing reduced blood flow through the gland. Reduction in blood flow has been shown to reduce the response to maximal secretory stimuli (Coats, Denton, Goding and Wright, 1956; Denton, 1957a). Other factors may be reduced rumenal activity, which in some instances can be accounted for by reduced food intake, and reduced activity of the secretory nucleus as evidenced by reduction of conditioned response to distance receptor stimuli (Denton, 1957c). Although in the non-depleted sheep the salivary Na^+/K^+ ratio does not change with changes in secretion rate (Denton, 1956), in Na^+ depletion there is a direct linear relationship between salivary secretion rate and Na^+/K^+ ratio. The position of the lines relating salivary Na^+/K^+ ratio to secretion rate changes as the Na^+ deficiency becomes greater.

When one increases the DOCA supplement of adrenalectomized sheep in normal Na^+ balance, the resulting fall in salivary Na^+/K^+ ratio is not accompanied by a fall in secretion rate, but when the Na^+ supplement is withdrawn, the further fall in salivary Na^+/K^+ ratio is invariably accompanied by a corresponding fall in secretion rate. Thus, the two factors so far recognized as being involved in the parotid response appeared to be dissociated. The relationship between salivary secretion rate and Na^+/K^+ ratio in adrenalectomized sheep during Na^+ depletion and repletion was studied in three groups of experiments. In group A, serial long-term (24 hr.) collections, a correlation was shown between saliva volume and Na^+/K^+ ratio. In group B, serial short-term (10-30 min.) collections, there were two categories of change of Na^+ balance: (i) slow changes (3-9 days) of Na^+ balance. There was an overall correlation between Na^+/K^+ ratio and secretion rate on each day of depletion, whether or not secretion was stimulated by ACh. (ii) Rapid change of Na^+ balance. The correlation between Na^+/K^+ ratio and secretion rate was unaffected by rapid correction of Na^+ deficiency. The results of groups A and B suggested that only two *major* factors appear to be contributory causes in the normal parotid response to Na^+ depletion. A third, quantitative, group of experiments, Group C, was undertaken with a view to determining whether any other factor was operative.

Group A. The relationship between salivary secretion rate and Na^+/K^+ ratio in serial 24 hr. saliva collections.

Examination of the results of the five experiments detailed in Section 1 (d) (Na^+ depletion while on constant increased DOCA dosage), showed that a linear relationship existed between the volumes of 24 hr. collections of saliva and the salivary Na^+/K^+ ratio during Stages C and D. Two typical experiments on P.F.20 are shown in Fig. 6. In the experiment shown in the top graph, the sheep received 417 m-equiv. $NaHCO_3$/day in the control period and the regression line showed a greater slope than in the experiment shown in the lower graph where it received 300 m-equiv. $NaHCO_3$/day in the control period. Both regressions were highly significant ($p < \cdot 001$). An important feature of the results was that this relationship held during the period of recovery from Na^+ deficit (closed circles on the graphs). This is not usually the case in a normal animal. In the normal animal (P.F.1—Fig. 7), restoration of the Na^+ supplement caused the salivary Na^+/K^+ ratio to rise rapidly as a result of inhibition of secretion of electrolyte-active steroid (Denton and McDonald, 1957), whereas the secretion rate returned to normal gradually over a period of 3-4 days. Thus the movement of the two factors was dissociated. In the experiments on the adrenalectomized sheep, where DOCA administration was constant throughout, the salivary Na^+/K^+ ratio rose slowly in direct relationship to the secretion rate. Thus, there was a difference between normal sheep and adrenalectomized sheep on

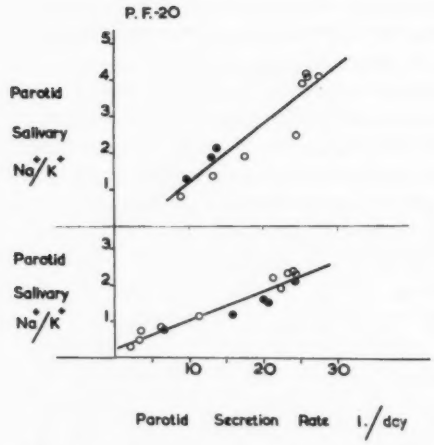

Fig. 6. *The relationship between parotid salivary Na+/K+ and daily secretion rate during two episodes of Na+ depletion in an adrenalectomized sheep* (*P.F.20*). DOCA dosage 20 mg./day throughout.

Upper graph: ○—3 days immediately before withdrawal of supplement (417 m-equiv. $NaHCO_3$/day) and 5 days' Na+ depletion. ●—Na+ replacement. $NaHCO_3$ supplement 417 m-equiv./day.

Lower graph: ○—3 days immediately before withdrawal $NaHCO_3$ supplement (300 m-equiv./day) and 7 days' Na+ depletion. ●—Na+ replacement. $NaHCO_3$ supplement 320 m-equiv./day.

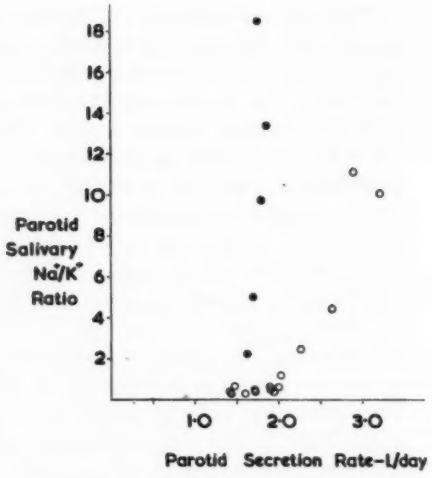

Fig. 7. *The relationship between parotid salivary Na+/K+ ratio and daily secretion rate during Na+ depletion in a normal sheep* (*P.F.1*). ○—2 days control period (600 m-equiv. $NaHCO_3$/day) and 13 days Na+ depletion. ● — Na+ replacement (600 m-equiv. $NaHCO_3$/day).

constant increased DOCA supplement, in their salivary response to Na^+ depletion. This suggested that the further fall in salivary Na^+/K^+ ratio which occurred in the latter may have been due solely to altered parotid secretory activity.

Group B. The relationship between salivary secretion rate and Na^+/K^+ ratio in serial short-term (10-30 *min.*) *saliva collections.*

(i) *Slow change of Na^+ balance, secretion rate stimulated by ACh.* As the effect of intramuscularly injected DOCA on salivary Na^+/K^+ ratio varied over a period of 24 hrs., it was first necessary to determine the interval of time after the injection of DOCA during which its action could be considered to be constant. Fig. 8 sets out observations made on P.F.20 during a period of Na^+ depletion whilst receiving 20 mg./day of DOCA. It can be seen that, in this instance, by 24 hrs. after the previous injection there was little residual DOCA action (−90 to −30 min.). There was a progressive fall in the salivary Na^+/K^+

ratio for 90-120 min. after the injection of the daily DOCA supplement of 20 mg. For the remaining 4 hrs. of the experiment there was a linear relationship between salivary Na^+/K^+ ratio and secretion rate. It was concluded therefore that, between the 2nd and 6th hr. after injection, the effect of DOCA was constant. Accordingly, the ACh infusion experiments described below were made at a fixed time (c. 4 hrs.) after the daily injection of DOCA.

The results of two experiments are shown in Figs. 9 and 10, and are described in detail below.

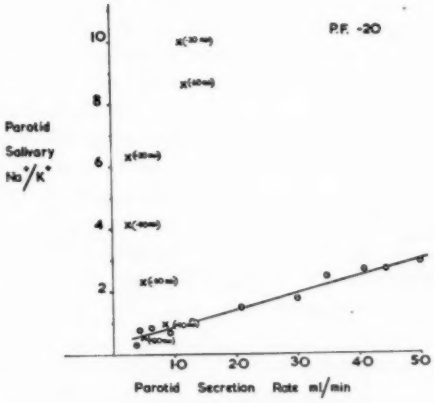

Fig. 8. *The effect of a single intramuscular injection of* 20 *mg. DOCA on the salivary Na^+/K^+ ratio of an adrenally insufficient Na^+-depleted sheep* (P.F.20). DOCA given at time 0. ×—First 210 min. of the experiment. ○—Collections made during the following 240 min.

In an experiment on P.F.20, the sheep had been given 300 m-equiv. $NaHCO_3$ daily during the control period and was given 20 mg. DOCA daily throughout the experiment. Fig. 9 (upper section) shows the effect on salivary Na^+/K^+ ratio of stimulation of the parotid gland by ACh infusion each day during a period of 7 days of Na^+ deficit. The linear regression of salivary Na^+/K^+ ratio on secretion rate for the whole period was highly significant ($p < \cdot 001$), though the Na^+ balance varied between equilibrium on the morning of withdrawal of supplement and a deficit of 590 m-equiv. on the 7th day. This is in contrast with the finding in a normal animal (P.F.40, lower section, Fig. 9) where the salivary Na^+/K^+ ratio at a specific secretion rate varied markedly with the state of Na^+ balance. On each succeeding day of Na^+ depletion there was a linear relation between secretion rate and salivary Na^+/K^+, the difference between the position of the lines on succeeding days presumably reflecting the changing titre of electrolyte-active steroid in the peripheral blood.

In a similar experiment on Adr. 8 (Fig. 10), the sheep was given 417 m-equiv. $NaHCO_3$ and 5 mg. DOCA daily in Stage A (Section 1 (d)). The Na^+ supplement was unchanged during Stage B, but the DOCA supplement was increased to 20 mg./day. As this animal had a lower salivary secretion rate than P.F.20, there was a much larger gain of Na^+ (+485 m-equiv.) during Stage B, and during this period, there was only a small fall in the salivary Na^+/K^+ ratio (24 hr. specimen) from 16·1 to 10·7. This is a further example of the way in which changes in Na^+ balance affect the salivary response to DOCA. At the end of Stage B (Day 0), and on six days during the Na+ de-

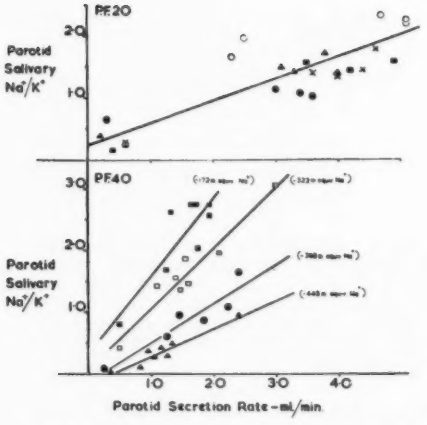

Fig. 9. *A comparison between the effects of short term stimulation of parotid secretion by ACh on successive days of Na+ depletion in adrenalectomized sheep* (*P.F.20*) *and normal sheep* (*P.F.40*).

Upper graph (P.F.20): ○—1st day of Na+ depletion. ●—2nd day of Na+ depletion. ×—4th day of Na+ depletion. ■—5th day of Na+ depletion. ▲—7th day of Na+ depletion.

Lower graph (P.F.40): ■—1st day of Na+ depletion. □—2nd day of Na+ depletion. ●—3rd day of Na+ depletion. ▲—4th day of Na+ depletion.

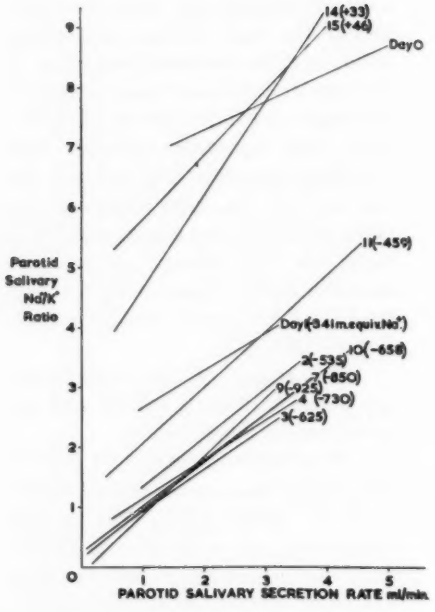

Fig. 10. *The effect of short term stimulation of parotid secretion by ACh on successive days of Na+ depletion and repletion in an adrenalectomized sheep* (*Adr.* 8). The $NaHCO_3$ supplement (417 m-equiv./day) was withdrawn on day 0, and replaced on day 9. For clarity, the individual points have been omitted. The figures in brackets indicate the Na+ balance relative to day 0.

pletion period, an ACh infusion was given at a period of 4-6 hrs. after the daily DOCA dosage. In this instance of an initial large positive Na^+ balance, there was a change in the position of the line relating secretion rate and salivary Na^+/K^+ after the first day of Na^+ deficit (Day 1, −341 m-equiv.). For the next eight days of Na^+ depletion, and on the first day of Na^+ replacement (Day 10), the lines relating secretion rate and salivary Na^+/K^+ overlapped. During this time there was a wide variation in the daily Na^+ balance, from −535 m-equiv. to −935 m-equiv. and return to −658 m-equiv. There was a significant linear relation between secretion rate and salivary Na^+/K^+ ratio for all 46 collections taken throughout this 9-day period ($p < \cdot 001$). On Day 11, when

the Na^+ deficit was — 459, the regression line moved close to that corresponding to the first day of Na^+ depletion. By Day 14 the sheep had regained the Na^+ lost during the Na^+ withdrawal period, and the regression lines were in a similar position to those relating to the end of the control period when the external balance showed a large Na^+ gain (+ 485 m-equiv.).

The salivary changes in this type of experiment again showed a clear-cut qualitative difference between the normal sheep, and the adrenalectomized sheep on constant increased DOCA supplement. In the latter, on every day of Na^+ depletion, the points on the graphs relating secretion rate to Na^+/K^+ ratio were on the same line. This feature held not only when the sheep was being deprived of Na^+, but also when the Na^+ was being restored to it. In the normal sheep, there was a different regression line for every day of progressive Na^+ depletion.

(ii) *Rapid change of Na^+ balance.* The contrast between adrenalectomized and normal sheep was also clearly shown when examining the response to rapid correction of Na^+ deficiency by a large intra-rumenal dose of $NaHCO_3$. A typical experiment was performed on sheep Adr. 8 (Fig. 11). This sheep was

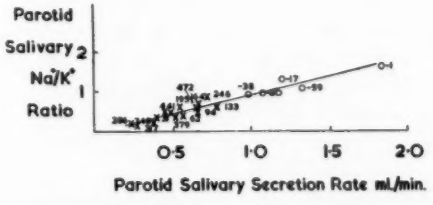

Fig. 11. *The effect on the relationship between parotid salivary Na^+/K^+ ratio and secretion rate of an intrarumenal dose of* 600 *m-equiv. $NaHCO_3$ during Na^+ depletion in an adrenalectomized sheep* (Adr. 8) *on constant DOCA supplement of* 20 *mg./day.* $NaHCO_3$ given at time 0. Observations made from — 80 to + 472 min.

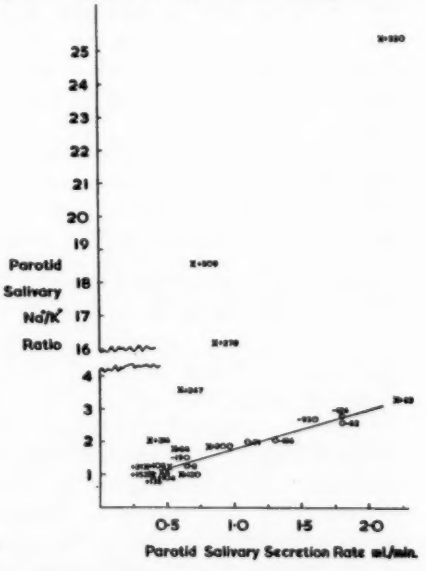

Fig. 12. *The effect on the relationship between parotid salivary Na^+/K^+ ratio and secretion rate of an intrarumenal dose of* 600 *m-equiv. $NaHCO_3$ during Na^+ depletion in a normal sheep* (George). $NaHCO_3$ given at time 0. Observations between — 156 and + 320 min.

given a constant DOCA supplement of 20 mg./day, and after withdrawal of its Na^+ supplement for three days it had a Na^+ deficit of 584 m-equiv. Serial saliva collections were made for two hours, and then the sheep was given 600 m-equiv. $NaHCO_3$ in 1·0 litre water by rumen tube. Further collections of saliva were made at ca. 30 min. intervals for the ensuing 443 min. 247 min. after the $NaHCO_3$ was given, 200 m-equiv. KCl was administered per rumen tube in order to offset the alkalosis and hypokalaemia which occurs in these circumstances. There is no evidence to indicate that, in a normal animal, such a procedure would prevent the normal response (Denton, 1956). There was a highly significant linear relationship ($p < \cdot001$) between the salivary Na^+/K^+ ratio and secretion rate for the whole period of the experiment. This contrasts with the response of a normal animal to rapid change of Na^+ balance, as shown by the sheep "George" (Fig. 12). After withdrawal of $NaHCO_3$ for four days the sheep had a Na^+ deficit of 650 m-equiv. Serial saliva collections were made for 186 min., and the sheep was then given 600 m-equiv. Na^+ in 1·5 l. water by rumen tube. There was a highly significant linear relationship ($p < \cdot001$) between salivary Na^+/K^+ ratio and secretion rate for the period before giving the $NaHCO_3$, and for 200 min. thereafter. From then on, this relationship between secretion rate and Na^+/K^+ ratio did not hold. A similar contrast is found between the responses of normal and adrenalectomized sheep to a rapid correction of Na^+ deficiency by the intracarotid route (Denton and McDonald, 1957).

In summary, the evidence from groups A and B indicates that only two major factors (an increased secretion of electrolyte-active adrenal hormone and diminished parotid secretory activity) appear to be contributory causes in the normal parotid response to Na^+ depletion.

Group C. The quantitative effect of ACh stimulation on salivary Na^+/K^+ ratio during Na^+ depletion on constant high DOCA dosage.

The effects of both factors referred to in the preceding summary can be held constant during an episode of Na^+ depletion. The effect of variation in electrolyte-active steroids can be eliminated in adrenalectomized sheep by giving constant increased DOCA supplement and making due allowance for time of action of DOCA. The effect of alterations in secretion rate can be eliminated by stimulating the parotid gland by ACh to a standard secretion rate. In these circumstances any change in salivary Na^+/K^+ ratio during Na^+ depletion would presumably be due to the operation of an additional factor.

A number of such experiments have been carried out. In some instances the intracarotid ACh infusion caused the salivary Na^+/K^+ ratio to return almost to the value pertaining to a specific secretion rate under the same DOCA dosage without Na^+ deficit, but in others it fell significantly short of this.

In a typical experiment on P.F.20, the sheep was given 20 mg. day DOCA supplement, and 417 m-equiv. $NaHCO_3$/day during the control period. The

salivary secretion rate was stimulated by intracarotid ACh infusion during this period, and in two subsequent episodes of Na^+ depletion. In the control period (secretion rate 4·0 ml./min.) the salivary Na^+/K^+ was $123/39 = 3·2$. After four days' Na^+ depletion, with a Na^+ deficit of 680 m-equiv., the salivary Na^+/K^+ was $98/51 = 1·0$ (4·1 ml./min.). Seven days later the Na^+ deficit was 600 m-equiv., but the salivary Na^+/K^+ was then $116/43 = 2·7$ (4·1 ml./min.).

In the experiments where ACh infusion did not raise the salivary Na^+/K^+ ratio to the control figure, the uncorrected residuum was due more to a failure of Na^+ to rise than of K^+ to fall. This may have been due to lowered plasma Na^+ concentration during Na^+ depletion. If NaCl were infused into the carotid artery concurrently with the ACh this residuum largely disappeared. For example, in another experiment on P.F.20 (DOCA 20 mg., $NaHCO_3$ 300 m-equiv./day), on the day before withdrawal of the Na^+ supplement the salivary Na^+/K^+ ratio was $115/59 = 2·0$. On the seventh day of depletion, at the same secretion rate, the ratio was $86/58 = 1·5$. On this day, 4 M NaCl was given by intracarotid infusion at 1·5 ml./min. (Denton and McDonald, 1957). The ratio then rose to 107/56-118/54 = 1·9-2·2. Two other experiments on P.F.20 also showed a similar result.

Hence the normal salivary response to Na^+ depletion is due not only to increased adrenal electrolyte-active steroid secretion and altered parotid secretory activity, but also in small part to the effect of lowered Na^+ concentration in the blood supplying the parotid gland.

Section 2.

The effect of Na^+ depletion upon other physiological functions.

The concentrations of the principal electrolytes in sheep's parotid saliva are Na^+ 180, K^+ 10, HCO_3^- 100-150, Cl^- 7-15 m-equiv./l. The pH is 8·3-8·6.

When considering Na^+ depletion produced by the drainage of parotid saliva, it should be recognized that the loss of Na^+ is far in excess of the loss of Cl^- relative to their proportions in the extracellular fluid (ECF). The Na^+ concentration is 15-30 times the Cl^- concentration. Thus a very severe stress is placed on the mechanisms concerned with regulating the ECF acid base balance, and the survival of the animal depends not only on the efficiency of the renal mechanisms for conservation of Na^+, but also on the mechanisms for increasing the excretion of Cl^- (Denton, Wynn, McDonald and Simon, 1951; Denton, 1957a).

The renal response to Na^+ depletion and the changes in the blood are described in sub-sections (a)-(d), corresponding to sub-sections (a)-(d) in Section 1. For convenience, the relevant changes in electrolyte balance and faecal composition are described separately.

(a) *Withdrawal of hormone supplement, Na^+ supplement continued.* Adrenal insufficiency was accompanied by an increased renal excretion of Na^+ and Cl^-. As the renal loss was greater than intake, a Na^+ deficit resulted. In Adr. 1 and P.F.20, there was a large diuresis of hypotonic urine. There was little change in the whole blood pH, and the plasma HCO_3^- concentration fell by only 2-4 m-equiv./l. The plasma Na^+ concentration fell to 125-138 m-equiv./l. and the K^+ concentration rose to 6·2-9·0 m-equiv./l. within 2-3 days (Goding and Denton, 1957). The plasma Cl^- concentration fell to 97-102 m-equiv./l.

Thus there was a fall in the osmotic pressure of the ECF without any definite disturbance of acid-base balance because the proportion of Na^+ to Cl^- lost in the urine did not differ greatly from that of their extracellular concentrations (Table 1).

(b) *Maintenance level of hormone supplement, Na^+ supplement withdrawn.* In these experiments, despite evidence of adrenal insufficiency in the impairment of the parotid response, and increase in the extracellular K^+ concentration, there was almost complete renal conservation of Na^+. In Adr. 8, the renal Na^+ excretion during Na^+ depletion averaged 2 m-equiv./day, and in P.F.20, it averaged 8 m-equiv./day. In P.F.20, there was a diuresis of 4-5 l./day and the urine contained only 1-2 m-equiv./l of Na^+. This diuresis, from 1·8 l./day in the control period to 5·1 l./day in the depletion period, occurred also in Adr. 1, but not in Adr. 8. In both experiments the renal excretion of Cl^- increased above intake, and a moderate Cl^- deficit resulted. In the experiment on P.F.20, the full ration of food was consumed each day, hence the Cl^- intake was constant. The average Cl^- excretion during the control period was 59 m-equiv./day, and rose to 88 m-equiv./day during Na^+ deficit. When the Na^+ supplement was restored, the Cl^- excretion fell to 25 m-equiv./day, indicating a retention of Cl^- to repair the body deficit.

In spite of this renal response, the extracellular acid-base equilibrium was disturbed, the degree of disturbance depending on the degree of Na^+ deficit. In Adr. 8, where a large Na^+ deficit resulted from the fistula loss, the pH of arterial whole blood fell from 7·40 to 7·16, and the plasma HCO_3^- fell from 30·1 to 14·8 m-equiv./l. In P.F.20, the rapid fall in secretion rate prevented a large Na^+ deficit, and the changes in pH and HCO_3^- were small, but in the same direction.

The plasma Na^+ concentration fell progressively in all experiments—in P.F.20 the fall was 17 m-equiv./l. in 3 days and, in Adr. 8, 25 m-equiv./l. in 5 days. The plasma K^+ concentration rose by 2·6 m-equiv./l. in P.F.20. The Cl^- concentration fell by 12 m-equiv./l. to 96 m-equiv./l. In the experiment on Adr. 8, there was a rise in plasma K^+ concentration of 4·0 m-equiv./l. to 8·2 m-equiv./l. over the first two days. There was then a fall to 6·0 m-equiv./l. on the 5th day. The plasma Cl^- concentration rose by 17 m-equiv./l. to 120 m-equiv./l.

on the third day and had fallen to 94 m-equiv./l. by the 5th day. During the period of fall in K^+ concentration there was no increase in K^+ loss from urine or saliva, in fact, there was a considerable decrease. There is insufficient information to provide an explanation of this.

Thus during Na^+ depletion on basal DOCA supplement there was evidence of adrenal insufficiency both in the failure of the parotid response and the changes in extracellular Na^+ and K^+ concentrations, but not in the renal mechanism for conservation of Na^+ and regulation of acid-base balance.

(c) *Graded increase of hormone supplement during a period of withdrawal of Na^+ supplement.* In this single experiment, renal conservation of Na^+ was again effective but, although Cl^- continued to be excreted by the kidney, the excess of output over intake was not sufficient to cause a significant Cl^- deficit (Table 1). There was much less disturbance in the composition of the ECF than in section (b). The plasma Na^+ concentration fell very little. The K^+ concentration remained in the range 5·3-6·35 m-equiv./l. except for the sixth day of Na^+ depletion when it was 8·4 m-equiv./l. The whole blood pH fell from 7·34 to 7·06 in two days and rose again to 7·30 by the end of the period. The plasma HCO_3^- concentration fell in two days also, from 24·0 to 13·7, and returned eventually to 22·5 m-equiv./l. The urine volume doubled on the first day of Na^+ depletion, but was normal thereafter.

Thus, when Na^+ depletion in an adrenalectomized sheep was "covered" by graded increase of hormone supplement, there was normal urinary conservation of Na^+ but a smaller Cl^- deficit than usual. The disturbance in the ECF was the same as that seen in the normal sheep.

(d) *Hormone supplement increased but constant before, during and after Na^+ withdrawal.* (This subsection includes data relating to subsection 1 (e).) In every experiment, Na^+ was conserved in the urine almost as effectively as in the normal sheep (Table 1). In the experiment described in Section 1 (e), withdrawal of the hormone supplement during a period of Na^+ depletion resulted in a large urinary loss of Na^+ (80 m-equiv.) within 24 hrs. There was a clear-cut fall in the urinary excretion of K^+ in five out of six experiments (Table 1). In practically all experiments, the kidney responded to the subtraction of Na^+ in excess of Cl^- by increased excretion of Cl^-, with a resulting negative balance of Cl^- (Tables 1 and 2).

The fall in plasma Na^+ concentration in Adr. 1 (DOCA 40 mg./day) was only 3 m-equiv./l., but in the remaining experiments, when the DOCA supplement was 20 mg./day, much larger falls in Na^+ concentration were recorded. In four experiments on P.F.20 the fall was 7-18 m-equiv./l. and in the experiment on Adr. 8 (9 days' depletion), 32 m-equiv./l. Blood pH and plasma HCO_3^- fell but no more than in a normal animal. The plasma K^+ concentration rose by 2-3·5 m-equiv./l. The maximum rise occurred at differing days of Na^+ depletion in the various experiments, e.g. on day 2 (2 experiments), day 4

TABLE 1.

The effect on electrolyte balance and daily urinary Na^+ excretion of episodes of adrenal insufficiency, Na^+ depletion on basal DOCA dosage, and Na^+ depletion on graded or constant increased dosage of DOCA.

Episode	Sheep	No. of days	Balance m-equiv.			Average daily Urinary loss of electrolytes m-equiv.					
						Na^+		K^+		Cl^-	
			Na^+	K^+	Cl^-	Control	Na^+ depletion	Control	Na^+ depletion	Control	Na^+ depletion
Adrenal insufficiency	Adr. 1	3	−136	+ 55	− 32	18	169	259	105	149	157
	P.F. 20	2	−310	+ 21	−192	5	233	86	198	71	157
Na^+ depletion on basal DOCA dosage	P.F. 20	3	−311	+ 47	− 75	4	8	125	152	59	88
	Adr. 8	5	−574	+ 24	−201	20	2	172	67	155	90
Na^+ depletion on graded increased DOCA dosage	Adr. 1	7	−439	+ 29	− 15	78	5	259	132	124	93
Na^+ depletion on constant increased DOCA	Adr. 1	6	−606	+309	−102	3	1	81	48	36	110
	P.F. 20	6	−515	+ 79	−179	1	1	28	3	31	53
	P.F. 20	4	−342	+188	+ 12	5	8	109	39	40	53
	P.F. 20	6	−605	+127	−136	4	6	40	79	35	67
	P.F. 20	5	−715	+ 56	−181	18	6	52	12	36	58
	Adr. 8	9	−925	+ 27	−154	67	1	139	13	120	58

TABLE 2.

The effect on electrolyte balance, daily urinary Na^+ excretion, and body weight, of withdrawal of the daily Na^+ supplement from an adrenalectomized sheep maintained on constant but increased dosage of DOCA.

Episode	Duration of episode (days)	DOCA dosage (mg./day)	Electrolyte Balance m-equiv.									Daily water balance (l.)	Urinary Na^+ (m-equiv/day)	Weight (kg.)
			Na^+			K^+			Cl^-					
			Intake	Output	Balance	Intake	Output	Balance	Intake	Output	Balance			
Control Period A	3	10	1092	932	+160	863	887	− 24	379	309	+ 70	+1·0	29	26·8
Control Period B Increase DOCA	5	40	1840	1481	+359	1517	1133	+384	665	324	+341	+1·5	3	29·9
Withdrawal of Na^+	6	40	430	1036	− 606	1865	1541	+324	819	920	−101	+0·8	1	27·5
Recovery Period A	3	40	1109	668	+441	933	965	− 32	409	268	+141	+0·9	1	30·1
Recovery Period B	4	40	1477	1157	+320	1239	1217	+ 22	544	360	+184	+0·9	1	31·1
Control Post Period	3	40	1109	1087	+ 22	933	931	+ 2	409	453	− 44	+0·8	34	31·4
Reduce DOCA	4	10	1478	1840	−362	1243	1734	−489	545	713	−168	+0·3	36	29·0

(2 experiments) and day 6 (2 experiments). Similar though less marked changes in plasma K^+ concentration occur in normal sheep (Denton, 1957d).

In the experiment on Adr. 1, the plasma Cl^- concentration fell by only 2 m-equiv./l. In four experiments on P.F.20, the falls ranged from nil to 11 m-equiv./l. In the experiment on Adr 8, the plasma Cl^- rose from 106 to 131 m-equiv./l. during the first 4 days of Na^+ depletion, and fell steadily to 104 m-equiv./l. over the following five days.

In every experiment Na^+ depletion was associated with an increase in urine volume. In three experiments there was a large diuresis, the average urine volume being 2½ to 5 times greater than during the preceding period on the increased DOCA supplement. An example of gradual increase of urine volume is well shown in Fig. 4. Since it occurred without significant impairment of the parotid or renal response, and was maximum when there was only a small change in plasma Na^+ or K^+, it would suggest that this water diuresis was an early indication of relative deficiency of adrenal hormones in some sheep.

Thus, during Na^+ depletion on constant increased DOCA, there was little or no impairment of renal regulation of acid-base balance.

Faeces. Relative to the large turnover of electrolytes involved in sheep with parotid fistulae, the usual faecal loss of Na^+ (c. 10-20 m-equiv./day) is small. Analyses of faeces made in the course of total balance studies showed that the loss/day of Na^+ increased during adrenal insufficiency, and decreased with increased DOCA supplement (Goding and Denton, 1957). Na^+ depletion resulted in almost complete conservation of Na^+ in every experiment when the DOCA supplement was 5 mg./day or above.

Electrolyte balance studies. An electrolyte balance during an experiment involving Na^+ deficit while the sheep received an increased but constant DOCA dosage is shown in Table 2. The result is typical of these experiments on P.F.20, Adr. 1 and Adr. 8, and the details will serve as a reference for other experiments quoted. The increase of DOCA dosage during the second period of Table 2 caused significant retention of Na^+ and Cl^- and also of K^+. This positive K^+ balance may be due mainly to replacement of some of the Na^+ in the rumenal digestive pool by K^+ as a result of increased K^+ concentration in the saliva (Goding and Denton, 1957). Upon withdrawal of the daily Na^+ supplement a considerable Na^+ deficit occurred, and renal activity also produced a negative Cl^- balance. There was a positive K^+ balance, which again may be accounted for by replacement of more of the Na^+ in the rumen by K^+ as a result of the further lowering of the Na^+/K^+ ratio of the saliva. These changes were reversed by replacement of the Na^+ supplement, and upon reduction of the DOCA dosage to basal there was a large negative balance of the three ions. The water balance became positive during the period of increased DOCA dosage and was negative upon DOCA withdrawal, and these changes were associated with weight gain and loss. The rather large daily positive water balance of ca. 1·0 l. under control conditions is assumed to indicate the

insensible loss during a summer month when the mean temperature was 70.4° F. Table 1 shows the changes in electrolyte balance produced by adrenal insufficiency, Na^+ depletion while a basal dosage of DOCA only was given, and Na^+ depletion whilst receiving graded increase of DOCA or constant increased DOCA. The effect on renal excretion of electrolytes is also shown in Table 1. In this table the control period relates to the period immediately preceding Na^+ depletion. In the experiments on increased DOCA, this corresponds to Stage B (Sect. 1 (d)) (increased DOCA, $NaHCO_3$ supplement continued).

DISCUSSION.

This study of the effect of Na^+ depletion on the salivary Na^+/K^+ ratio of bilaterally adrenalectomized sheep was undertaken in an endeavour to elucidate the role of the adrenal cortex in the parotid response to Na^+ depletion in the normal sheep.

It was found that the parotid response to Na^+ depletion did not occur when the Na^+ deficit was caused by renal loss of Na^+ due to adrenal insufficiency. Also, there was little or no parotid response when a Na^+ deficit was produced by withholding the daily Na^+ supplement from a sheep receiving only the usual daily maintenance dose of DOCA. Despite a very large decrease of secretion rate, the salivary Na^+/K^+ decreased only a small amount and, in one instance, rose again on the third day. This was associated with the characteristic signs of adrenal insufficiency in the sheep (Goding and Denton, 1958). However, if the sheep were given a step-wise increase of DOCA dosage during Na^+ depletion, the parotid response was similar to that of a normal sheep. These experiments established that an increased secretion of the adrenal cortex was essential for the normal parotid response to Na^+ depletion.

If the adrenalectomized sheep were given an increased but constant dosage of DOCA for 8-10 days, an equilibrium state with a considerably lowered salivary Na^+/K^+ ratio resulted, and if the daily Na^+ supplement were withdrawn then, the salivary Na^+/K^+ ratio decreased to the level seen in Na^+ depletion in a normal sheep. Restoration of the Na^+ supplement caused the ratio to rise again to that resulting from the increased but constant daily dosage of DOCA.

This experiment, in which variation of adrenal steroid action was excluded, like the control experiment in which graded increased dosage of hormone was given without concurrent production of Na^+ deficit, indicated that one other factor at least, in addition to the adrenal cortex is involved in the parotid response to Na^+ depletion in the normal sheep. The experiments in which adrenal hormones were withheld for one day during the course of Na^+ deficit, like the experiments on uncomplicated adrenal insufficiency and Na^+ deficit on basal DOCA dosage, confirmed that increased secretion of electrolyte-active hormone by the adrenal was essential if the action of any other causal factor was to be apparent.

In a normal animal a local parotid factor related to secretion rate fall is responsible for some 20-50 p.c. of the Na^+/K^+ ratio decrease during Na^+ deficit. The effect can be reversed by ipsilateral intracarotid infusion of ACh (Denton, 1957b). On the question as to whether this factor was involved in the parotid response in the adrenalectomized sheep, and whether it is the only other factor in addition to the adrenal involved in the normal animal, the following evidence emerged. The experiments on Na^+ deficit and its correction whilst administering increased constant DOCA dosage showed a significant linear relation between daily secretion rate and salivary Na^+/K^+ ratio. Usually this did not hold in a normal animal, presumably because of variation of adrenal secretion in response to the changes of Na^+ balance.

The experiments involving ACh infusion into the Na^+-depleted, bilaterally adrenalectomized sheep maintained on constant increased DOCA were not decisive in that the infusion did not consistently raise the salivary Na^+/K^+ ratio to the equilibrium pertaining to the increased constant DOCA dosage prior to Na^+ deficit. This, in part, may be attributable to some change of sensitivity of the parotid to a specific daily dosage of DOCA during transition from a state of positive Na^+ balance to one of Na^+ deficiency. It has been shown (Goding and Denton, 1957) that when a sheep was given 60 mg/day of DOCA concurrently with a very high supplementary Na^+ intake (595 m-equiv./day) the DOCA had very little effect on the salivary Na^+/K^+. Moreover, in the sheep P.F.20, the "equilibrium" Na^+/K^+ caused by 6 days' DOCA dosage of 20 mg/day was lower when the sheep received 298 m-equiv./day Na^+ supplement than when it received 417 m-equiv./day. However, once a small Na^+ deficit was established, there was no evidence (Fig. 10) that progression of deficit from 200 to, for example, 800 m-equiv. caused any further change of sensitivity. The change of sensitivity was apparent only between positive Na^+ balance and a state of mild deficiency. Examination of the results also suggested that the salivary Na^+ concentration played the larger part in the residuum not corrected by ACh infusion. Na^+ deficit in such an animal involved a decrease of the Na^+ concentration of the plasma perfusing the gland, and a change in the electrolyte concentrations in the parotid tissue itself. It is possible that the local action of these two factors, along with some increase in parotid sensitivity to the daily dose of DOCA (particularly in the instances where a high Na^+ load was given in the control period), was responsible for the uncorrected residuum. This was supported by the results of the slow 4 M NaCl infusions made concurrently with the ACh infusion. The evidence indicates that there are two major factors only in the parotid response to Na^+ deficit in the normal sheep, viz. an increased secretion of electrolyte-active adrenal steroid, and a local parotid factor related to decrease of secretion rate. The results here suggest also that the finding in *normal sheep* that DOCA injection caused a fall of salivary Na^+/K^+ ratio during slight or moderate Na^+ deficit, whereas the same dose had no effect during gross Na^+ deficit (Goding and Denton, 1956),

is explicable on the basis that, in the latter instance, the parotid gland was already responding maximally to endogenous secretion of electrolyte-active steroid.

Consideration of the parotid and renal responses to Na^+ deficiency in adrenalectomized sheep supported by varying dosage of DOCA is directly relevant to the discussion which has taken place between Selye (1954, 1955) and Ingle (1954) on the terms "permissive" and "conditioning" as applied to hormone action. Selye (1954) states: "Supposing a stimulus acts on a target organ in the presence, but not in the absence, of an endocrine gland, two possibilities exist:

(a) The stimulus acts directly on the target organ but only if the latter is maintained in a responsive condition by constant hormonal impulses which it receives from the gland.

(b) The stimulus has no direct effect upon the target but merely induces the gland to release a hormone which in turn acts upon the target".

The results in this paper show that both processes are involved in the total response of the sheep's parotid to Na^+ depletion in that—

(i) in a Na^+-depleted sheep, a local parotid factor associated with secretion rate lowers the salivary Na^+/K^+ ratio;

(ii) Na^+ depletion causes the adrenal gland to release a hormone which acts upon the parotid to lower the Na^+/K^+ ratio.

In describing the role of the adrenal in this physiological response to Na^+ depletion, it is clear that in (ii) the term "permissive" would not be applicable, since *the adrenal itself* is directly responsible for a large amount of the salivary change. In this case, the correct description of the adrenal role is that of a contributory condition in a set of jointly sufficient and severally necessary conditions for the response to occur or, for brevity, it might be referred to as a "contributory cause" of the response. With (i), ***this relation did not hold in an adrenally insufficient sheep,*** but when the adrenalectomized sheep on basal DOCA dosage became Na^+-depleted there was a fall of salivary Na^+/Ka^+ ratio in response to decrease of secretion rate, though it was not as large as usual relative to the degree of fall of secretion rate which occurred. The results suggested that, whereas decrease of secretion had an effect when basal dosage only of DOCA was given, it required greater than basal amounts of the hormone for this factor to produce the full range of its effect. The role of the adrenal here approximates to that of a "permissive" condition. The fact that it is necessary that more than basal DOCA be given for the local parotid factor to be fully manifest may not have indicated an increased local requirement of the parotid so much as an increased utilization and requirement in the whole body. Hence, increased injection may have been required to give the usual local parotid threshold amount "permissive" of the response.

By contrast, the adrenal role in the renal response to Na^+ subtraction briefly referred to in the "Results" section would appear to accord strictly with

Ingle's term "permissive". When a large volume of fluid which contains 10-20 times as much Na^+ as Cl^- is withdrawn from the extracellular fluid, the maintenance of an acid-base balance compatible with life depends to a large extent on a renal mechanism involving complete conservation of Na^+ and an increase in the rate of Cl^- excretion. The kidney depletes the animal of Cl^- considerably in excess of any Cl^- loss from the fistula (Denton, 1949; Denton, Wynn, McDonald and Simon, 1951; Denton, 1957a). However, if a sufficient minimum dosage of DOCA were given to an adrenalectomized sheep in these circumstances, Na^+ was retained and the increased Cl^- excretion occurred. The Cl^- excretion mechanism was independent of DOCA dosage and was quantitatively determined by the degree of Na^+ subtraction from the extracellular fluid. Whereas under control conditions with normal Na^+ balance increased DOCA dosage changed parotid saliva in a manner similar to Na^+ deficiency, its effect on the kidney was to cause retention of both Na^+ and Cl^- – that is, it did not reproduce the renal response seen when extracellular Na^+ is subtracted in excess of Cl^-. Hence, this renal response is not *regulated* by the DOCA level. The action of DOCA could, at the most, be described as "permissive" of this renal response to excess Na^+ subtractions but in the case of the parotid it is a "contributory cause" of the response to Na^+ depletion. Selye (1954), writing on the use of these terms, states: "The possibility cannot be refuted that such 'permissive actions' may play a role in stress responses but we would be hard-pressed to cite a clear-cut example", but in 1955 he states: "Actually it is precisely in the specific and not in the non-specific (stress) reactions that the corticoids play a purely permissive rôle of this type Here they are necessary only to prevent stress and collapse, thus keeping the tissues responsive". It is interesting in our experiments, where the "stress" on the organism is a defined disturbance of the extracellular electrolytes, that the role of the adrenal in body fluid homeostasis appears to be "permissive" of the action of one electrolyte regulation, and a "contributory cause" of the action of another.

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HAGEMAN FACTOR AND CAPILLARY PERMEABILITY

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SUMMARY.

A factor which increases capillary permeability appears in human plasma when this is exposed to a glass surface. The reaction did not take place in plasma deficient in Hageman factor or in "component A" both of which are necessary for the release of plasma kinin. Other similarities of these two sets of reactions also suggest that permeability factor and plasma kinin represent different stages of the same process.

INTRODUCTION.

Ratnoff and Colopy (1955) described a congenital condition characterised by a grossly prolonged clotting time *in vitro* in the absence of any haemorrhagic symptoms. They named the factor lacking in the plasma of such persons "Hageman factor". Hageman factor (HF) appears to be responsible for the initiation of a series of reactions when plasma is exposed to foreign surfaces among which glass is one of the most active. Some of these changes lead to coagulation (Margolis, 1956, 1957a, b; Shafrir and de Vries, 1956; Biggs *et al.*, 1958), others to the release of plasma kinin (probably a polypeptide) which contracts plain muscle and produces pain (Armstrong *et al.*, 1957; Margolis, 1957b, 1958b).

Other authors have shown that a globulin factor which increases capillary permeability appears in animal and human plasma after incubation in a high dilution with physiological saline (Miles and Wilhelm, 1955; Spector, 1957; Stewart and Bliss, 1957). The formation of a permeability factor by contact with glass has been demonstrated in guinea-pig plasma (Margolis, 1958a). The results suggested a close correspondence of this phenomenon with the changes leading to the release of kinin, but an experiment with human material was required in order to evaluate these findings in the light of previously established information.

The object of this paper is to demonstrate the activation of a permeability factor in human plasma by contact with glass and to investigate the possibility that this is another aspect of the same reaction that leads to blood clotting and the release of plasma kinin.

Three well-defined stages can now be recognised in the formation of plasma

kinin. They can be accounted for by the following hypothesis. HF is adsorbed onto the glass surface where it acts catalytically converting another component in plasma ("component A") into an active agent. This in turn interacts with "component B" to release plasma kinin (Margolis, 1957b, 1958b, c). Component B is rapidly consumed in this reaction. Component A is progressively exhausted by continued exposure to glass. HF is not used up in the same sense as the other components, but is simply removed by adsorption which is almost instantaneous. The loss of HF from plasma is therefore dependent mainly on the surface area of contact and not on the time of exposure. By graduated contact with glass it is possible to prepare plasma which is depleted of component B only, or in both components A and B (see Methods). The amount of surface sufficient to attain the latter is still too small to remove more than a small fraction of the HF contained in plasma. Since the products of the contact reaction are rapidly inactivated in plasma, the above preparations become biologically inert after a period of incubation in siliconed tubes and they can be used as a starting material for further experiments.

Note on terminology: The term "contact factor" has been formerly applied to the first detectable product of the contact reaction in the plasma (Margolis, 1957a, b; 1958b). As additional stages of this reaction were revealed, the meaning of this term had to be modified. In the meantime other authors have adopted this term as an equivalent to HF (Caen and Bernard, 1958; Soulier *et al.*, 1958). But the activated HF now appears to be present only on the glass surface and not in the supernatant plasma (Margolis, 1958c; Hardisty and Margolis, 1959). Perhaps these contradictions could be resolved by introducing such names as "fixed contact factor" and "soluble contact factor(s)", but it is probably better to wait until an agreed usage of the term is established. It will therefore not be used in the present report.

Methods.

Unless otherwise stated, plasma was handled in siliconed glassware (= "intact plasma").

Human citrated plasma was prepared as previously described (Margolis, 1957a). Samples of normal plasma and that from the patient with Hageman trait (*cf.* Biggs *et al.*, 1958) were freeze-dried in siliconed ampoules and stored *in vacuo* at — 20° C. These were reconstituted with distilled H_2O on the day of the experiments and the pH was adjusted to that of fresh plasma by adding N/10 HCl. Fresh normal plasma was also tested in parallel.

Plasma depleted of component A (and B) was prepared by rotating the normal samples with 1 gm./ml. of glass Ballotini beads (microspherules 0·1 mm. diam.) for 1 hour. The plasma was then transferred into siliconed tubes and left at room temperature for 4 hours.

Plasma depleted of component B. Normal plasma was rotated for 2 min. with 1 gm./ml. of beads. It was then removed and immediately mixed with an equal volume of intact plasma. The mixture was incubated in siliconed tubes for 4 hours at room temperature. This treatment results in the "consumption" of component B but the plasma is still rich in component A (*cf.* Margolis, 1958b).

Contact activation of plasma. In most tests the plasma samples were diluted with 3 volumes of 0·9 p.c. NaCl solution immediately before use. 0·8 ml. volumes were then exposed for 3 min. to approx. 140 $cm.^2$ of glass surface by rotating with 0·7 gm. of beads. The beads were allowed to settle (10-20 sec.) and the samples tested without delay. (The preliminary dilution of plasma is not an essential step but it gives somewhat clearer results,

probably because the concentration of citrate is lowered (*cf.* Spector, 1957) and also because decay of the active products is slowed (see Table 1: tests 3, 4, 6, 7 and Discussion).)

Test procedure (Stewart and Bliss, 1957). The subjects were "blued" by an intravenous injection of 70 mg. of Evans blue (T 1824) in 14 ml. of saline. 0·1 ml. volumes of the test plasma were injected intradermally into the anterior surfaces of upper arms and fore-arms using siliconed "tuberculin" syringes and No. 26 gauge needles. The resulting blue lesions were measured 20 min. after injection.

RESULTS.

The experiments reported here have been preceded by a number of preliminary tests on different subjects. These were performed without the administration of blue dye. Even so the differences between positive and negative results, assessed by the character of the wheals, were sufficiently clear to serve as a guide for the design of definitive experiments.

The injection of glass-activated, normal whole or "B-depleted" plasma produced blue lesions between 10 and 15 mm. in diameter, which began to appear in about 3 min. and increased in size and intensity for another 15-20 min. (Table 1: tests 2, 3, 6.) Control "non-contacted" plasma did not give rise to any blueing except at the site of the skin puncture (tests 1, 5, 9). Identical results were obtained with fresh and freeze-dried plasma.

TABLE 1.

The results of intradermal injections of 0·1 ml. volumes of plasma in "blued" subjects.

Test sample	Nature of injected plasma (O) = "intact"; (G) = activated with glass (see Methods).	Diameter (mm.) & intensity of blue lesions		
		Subject 1 (duplicate tests)		Subject 2
1	Whole (O)	—*	—	—
2	Whole (G)	13+++	12+++	12+++
3	Depleted of component B (undiluted**) (G)			11++
4	Sample 3**, after incubation at 22° C. in a "Polythene" tube for 6 min.			7+
5	Depleted of component B (O)	—	—	—
6	Sample 5 (G)	12+++	11+++	12+++
7	Sample 6, after incubation at 22° C. in a "Polythene" tube for 6 min.			10++
8	Whole, pre-heated to 60° C. for 20 min. (G)			—
9	Whole, pre-heated to 56° C. for 20 min. (O)			—
10	Sample 9 (G)			10+++
11	Hageman factor—deficient (G)	—	—	
12	Depleted of components A & B (G)	—	—	
13	Equal parts of 11 & 12 mixed before activation (G)	12+++	12+++	
14	Equal parts of 11 & 12 mixed after activation (G) in separate tubes	—	—	

*Pin-point blueing at the site of skin puncture.

**Except in tests 3 & 4 all samples were diluted with 3 parts of saline immediately before use (see Methods).

In agreement with the previous findings in guinea-pigs (Margolis, 1958a), pre-heating of plasma to 56° C. did not prevent activation but heating to 60° C. abolished the reaction (tests 8, 10).

When activated plasma was transferred into "Polythene" tubes the activity rapidly decayed. The decay appeared to be slower in the samples diluted with saline than in full strength plasma (tests 4, 7).

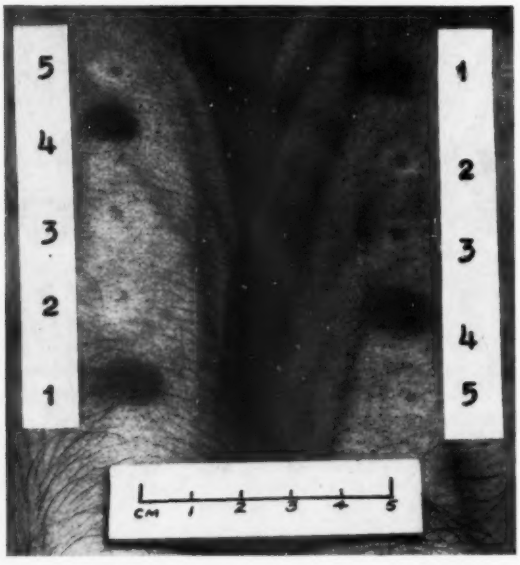

Fig. 1. Intradermal injections of plasma (diluted 1:4). Photograph taken 25 min. after the administrations. Duplicate tests. (1): HF-deficient plasma treated with glass which was "coated" with normal plasma (see text). (2): As (1) but treated with clean glass. (3): Normal plasma, depleted of components A and B treated with glass. (4): Mixture in equal parts of (2) and (3) treated with glass together. (5): As (4) without glass treatment. (See text and Table 1).

Plasma deficient in HF did not produce any response when treated with bare glass (Table 1: test 11; Fig. 1: test 2), but it could be fully activated by beads which had been previously exposed for 2 min. to normal plasma (either whole or depleted of components A and B) and then washed with 10 changes of saline (Fig. 1: test 1). Glass-treated plasma depleted of components A and B did not itself increase permeability (Table 1: test 12; Fig. 1: test 3), but it corrected the defect of HF-deficient plasma. The mutual correction took place only if the two plasmas were mixed first and activated together (Table 1: test

13; Fig. 1: test 4) but not when they were separately exposed to glass Ballotini and mixed afterwards (Table 1: test 14). Non-contacted samples of the mixture gave negative results (Fig. 1: test 5) showing that the reaction was specific to contact and was not an unrelated phenomenon (Margolis, 1958c).

DISCUSSION.

The above results show that a permeability factor is formed in human plasma as a result of contact with glass. This reaction takes place under the following conditions: (1) The HF must be present, since HF-deficient plasma did not produce the permeability factor. (2) Plasma treated to remove "component A" does not increase capillary permeability. The critical range of heat inactivation and the rapid rate of decay (which have been previously noted in relation to the release of plasma kinin) also suggest that component A is necessary for the formation of the permeability factor. (3) Plasma deprived of "component B" was as effective as whole plasma. Since component B is essential for the release of plasma kinin, the kinin does not appear to contribute significantly to the permeability-increasing effect of "contacted" plasma.

The present findings (see Table 1: tests 13, 14; Fig. 1: test 1) also give further support to the conclusion that the activated HF is fixed to the glass surface (Margolis, 1958c; Hardisty and Margolis, 1959).

In previous work (Miles and Wilhelm, 1955; Stewart and Bliss, 1957) a permeability factor has been produced by high dilution with physiological saline and not by a deliberate exposure to glass. It seems possible that this is identical with that demonstrated in the present experiments because: (a) in the earlier work glass containers were used; (b) when the dilution experiments were repeated in siliconed or "Polythene" containers the results were in no way comparable to those performed in glass tubes (unpublished observations in collaboration with A. A. Miles). It is possible that dilution *per se* does not activate the permeability factor but merely slows its destruction and thereby renders the plasma a more sensitive indicator of surface activity. The results of an investigation (in collaboration with C. A. Keele and V. Eisen) to be published elsewhere show that the effect of dilution on the release of plasma kinin (Schachter, 1956) can be explained in this way. Although the permeability factor and plasma kinin are not the same they seem to represent different products of the same chain of reactions. At least they are both initiated by HF and are probably also linked at the next stage through component A. In so far as these systems share a common mechanism, any inferences that apply to the release of plasma kinin should also be valid to the formation of permeability factor. The simplest hypothesis which would account for the close correspondence between the features of these two sets of phenomena would be that the activated component A (previously called "contact factor") and the permeability factor are the same substance.

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ANAMNESTIC RESPONSES IN RABBITS IMMUNIZED WITH TWO RELATED BACTERIAL VIRUSES

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SUMMARY.

Using T2 and T4 as two related antigens the secondary response of rabbits to the homologous and heterologous phage has been studied.

When T2 is followed by T4 there is a rise in anti-T2 somewhat less than that of anti-T4. The character of the antibody is, however, unchanged, absorption with T2 removing all antibody while that with T4 left a substantial residuum of specific anti-T2. The same occurred when T4 was followed by T2.

No evidence was found of the production of specific antibody being produced to an antigen on the injection of another related one. There is only a marked increase in the same type of cross-reacting antibody as was produced at the primary immunization.

INTRODUCTION

Recent studies on the response of animals previously immunized with and producing antibody against one antigen, when they are inoculated with a second, indicate that a secondary type response is obtained only when the second antigen is demonstrably related to the first, in the sense that cross-reaction will occur of one antigen with "pure" antiserum against the other antigen. The implications of anamnestic responses for immunological theory are important and extensive work has been reported: (a) on the reactions of human and bovine serum albumins as antigens in rabbits (Dixon and Maurer, 1954), and (b) on the effects of repeated infection or immunization with influenza virus antigens in man and in experimental ferrets (Davenport and Hennessey, 1956; Davenport *et al.*, 1953; Jensen *et al.*, 1956).

Working with human (HSA) and bovine (BSA) purified serum albumins Dixon and Maurer found that the second antigen provoked a secondary type response with, in addition to a large increase in the amount of cross-reacting antigen, production of more specific homologous antibody as well as some specific heterologous antibody. Davenport and his associates in an extensive series of papers have stressed the fact that amongst human beings the type of antibody against influenza A viruses that predominates in the serum is most likely to be that corresponding to the strains of virus that were current during the individual's childhood. The suggestion supported by both observational and

experimental evidence was that subsequent infections or immunizations provoked an antibody response of the same type as the original infection.

In experiments with ferrets serially immunized with three distinct types of influenza A virus, Jensen *et al.* (1956) uncovered a different pattern of response. When strains A, B and C were given at intervals in that order there was a progressive increase in the width of reactivity so that after the third infection antibody to all three strains was of comparable titre. This serum when absorbed with A lost all antibody against A, B and C, absorbed with B or C much of the anti-A remained.

Bacteriophages T2 and T4 are known to be serologically related (Delbruck, 1946). They produce high titre antiserum in rabbits whose antibody content can be accurately estimated in terms of the rate of phage neutralization and seemed to offer attractive material for studies in this field.

The chief feature of the results reported is a demonstration that these viral antigens behave very similarly to the influenza viruses used in Jensen's experiments. No evidence was found of a true anamnestic response, i.e. a stimulation by the second antigen of a specific antibody to the first.

Materials and Methods.

Phage stocks. Bacteriophages T2r and T4 were obtained from Dr. Bruce Holloway and grown on a strain of *Shigella flexneri* (YS, Hiss and Russell). Methods used followed those of Adams (1950), the lysates being prepared by the broth method. They were spun at 5,000 r.p.m. for 30 minutes to remove the unlysed bacteria and heated at 56° C. for 30 minutes to kill any remaining bacteria. Lysates containing approximately 2×10^{10} phage/ml. were used routinely and stored at 4° C.

Sera. Young adult rabbits were used. Four injections of 5 ml. of lysate over 10 days were given intravenously as the primary stimulation. Sera obtained 14 days after the last injection were titrated and shown to possess antibody but were not used in the experiments reported in this paper. Three months after the beginning of the primary injections a single secondary injection of lysate was given intravenously on Day 0. The rabbits were bled at regular intervals from the ear vein after this injection. All sera were heated at 56° C. for 30 minutes and stored in ampoules at − 12° C. until required. Samples under test were diluted as required in nutrient broth and kept at 4° C. over the period necessary to complete an experiment. Under these conditions the serum titre showed no detectable change in 10 days.

Phage-coated bacteria. These were prepared by the method of Burnet (1933). The bacteria were washed from overnight nutrient agar plates and heated at 56° C. for 40 minutes. By adding 2×10^{10} heat-killed bacteria to 15 ml. of lysate and keeping at 45° C. for 1 hour 99 p.c. of the phage was absorbed. By repeating this three times it could be assumed that between 30 and 40 phage particles were absorbed to each cell. After the last centrifugation the bacteria were taken up in broth containing 0·25 p.c. formalin and kept at 45° C. for 40 minutes to inactivate any free phage. They were then spun down again and resuspended in broth at a concentration of 5×10^{10}/ml.

Absorption of sera. The first absorption experiments soon showed that the wide range of antiphage titres of the sera would make it necessary to adjust the titre of the stronger sera by appropriate dilution to about the level of the lower titre material. This would then allow the use of a standard absorbing dose of phage-coated bacteria throughout. In each case the titre considered in making the primary dilution was that against the phage being

used for the absorption. As an example of the procedure adopted Table 1 shows the dilutions used with sera from the rabbits given T2 followed by T4.

TABLE 1.

To illustrate procedure of standardising serum titres.

Rabbit	Original titres K		Dilution used for absorption with	
	T2	T4	T2	T4
1185				
Day 0	102	99	1	1
Day 7	515	770	5	7
1187				
Day 0	460	210	4·6	2
Day 7	656	1050	6·5	10

These preliminary serum dilutions were then absorbed first with heat-killed bacteria YS, and then with phage-coated bacteria, the procedure in outline being as follows:

1 ml. primary serum dilution was mixed with 4 ml. heat-killed YS bacteria (5×10^{10}/ml.). This was held at 45° C. for 40 minutes, left overnight at 4° C. and then given a final 10 minutes at 45° C. before centrifugation. Copious agglutination occurred.

To the absorbed sera an equal volume of phage-coated bacteria, 5×10^{10}/ml., was added and the same sequence followed. Again there was copious agglutination. The final supernatant represented a 1:10 dilution of the starting material.

Antiphage titrations. The procedure used followed in general that described by Adams (1950). In titrating unabsorbed sera an appropriate number of dilutions, usually 4 fold but if necessary 2 fold, were made in broth and a volume of 0·45 ml. was used. To each tube 0·05 ml. of phage 5×10^6/ml. was added and the mixture held at 45° C. for 30 minutes. From this 0·05 ml. was removed and diluted 100 and 1000 times in broth. These were plated in 0·02 ml. amounts on the surface of plates spread with YS. For control platings the stock 5×10^6/ml. phage was diluted 10^{-4} before plating to give approximately 10 plaques per 0·02 plated. The plates used were nutrient agar containing 0·1 p.c. gentian violet.

The amounts of absorbed sera available were small and the following modification was used. Phage diluted to 10^3/ml. and an equal volume (0·05 ml.) of diluted serum were mixed and held at 45° C. for 30 minutes. Then 1 ml. of 0·7 p.c. nutrient agar at 40° C. containing YS from a broth culture in logarithmic phase of growth was added and the whole poured onto the surface of a nutrient agar plate. Control counts were made from phage-broth mixtures diluted 1:10 before the final pouring. Each series of titrations was done with a single phage lysate so as to avoid any differences in serological affinity between different batches (Streisinger, 1956).

All results are expressed as velocity constants using K^{45} to differentiate them from the values obtained at 37° C. by Adams' method.

RESULTS.

Homologous and heterologous secondary stimulation.

The antibody responses of eight experimental rabbits are shown in Figs. 1-4 as tested with both phage antigens. Four animals received the homologous antigen as a secondary stimulus, four the heterologous. In all cases both titres

show a logarithmic increase reaching a maximal titre on the 7th day, i.e. a typical secondary response is given by a related as well as by the homologous antigen.

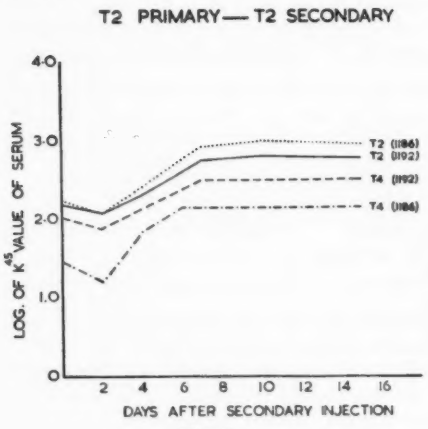

Fig. 1.

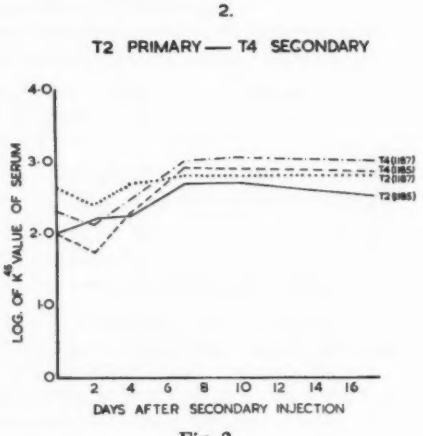

Fig. 2.

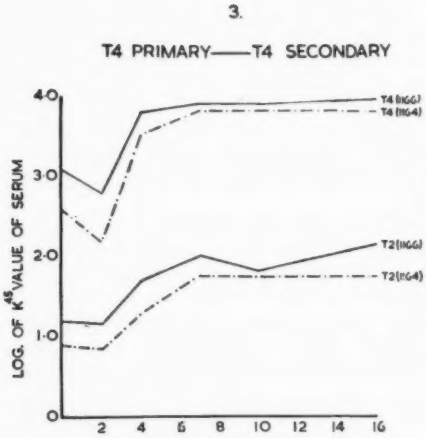

Fig. 3.

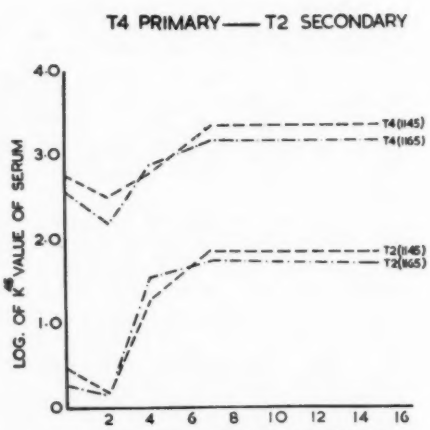

Fig. 4.

As maximal titres were reached on Day 7 sera taken on this day were used for all detailed comparisons and absorptions. Sera from Day 0 were used when required as controls. Tables 2 and 3 compare the titres obtained with each set of Day 0 and Day 7 sera. Each value is the mean of three separate titrations.

TABLE 2.

Comparison of day 0 and day 7 titres of rabbits receiving a T2 primary stimulation.

Serum	Secondary injection	T2 titres			T4 titres			T4 increase / T2 increase
		Day 0	Day 7	Day 7/ Day 0	Day 0	Day 7	Day 7/ Day 0	
1185	T4	102	515	5·0	99	770	7·9	1·5
1186	T2	180	850	4·8	28	139	4·9	1·0
1187	T4	460	656	1·4	210	1050	5·0	3·4
1192	T2	158	582	3·6	89	310	3·5	1=·0

The titres of the serum are the K^{45} value calculated from

$$K^{45}=2\cdot3\frac{\text{dilution of serum}}{\text{t (reaction time)}}\times\log=\frac{\text{original phage}}{\text{remaining phage}}$$

for cases where the neutralization was between 90-99%.

TABLE 3.

Comparison of day 0 and day 7 titres of rabbits receiving a T4 primary stimulation.

Serum	Secondary injection	T2 titres			T4 titres			T4 increase / T2 increase
		Day 0	Day 7	Day 7/ Day 0	Day 0	Day 7	Day 7/ Day 0	
1145	T2	3	72	24·0	518	2300	3·5	6·8
1164	T4	7·7	77	10	385	6436	16·6	0·76
1165	T2	1·9	56	29·4	372	1533	4·1	7·2
1166	T4	15	100	6·6	1770	8320	7·1	0·96

K^{45} values of serum as calculated from

$$K=2\cdot3\times\frac{\text{dilution of serum}}{\text{t (reaction time)}}\times\log\frac{\text{original phage}}{\text{remaining phage}}$$

for cases where neutralization is 90-99%.

The chief features of these Tables are: (1) T2 alone provokes antibody of the same order of activity against T2 and T4. (2) T4 alone produces only very small amounts of anti-T2 compared with the titre against T4. (3) In each case the heterologous secondary stimulus results in a change of the ratio in favour of the heterologous antigen though there is considerable variation amongst the animals. The change is most clearly seen in the rabbits given T4 followed by T2.

The results of the absorption experiments are shown in Tables 4 and 5. In Table 4 sera from rabbits initially immunized with T2 and given T4 as a secondary stimulus are shown. Absorption of these sera with T2YS removed all antibody against T2 and T4. Absorption with the heterologous phage left a substantial residuum of specific anti-T2 without significant indication of any difference resulting from the heterologous secondary stimulus.

TABLE 4.

Absorption of sera which had a T2 primary injection followed by T4.

Sera	Absorbed with T2YS		Absorbed with T4YS	
	Residual T2	Residual T4	Residual T2	Residual T4
85 Day 0	<0·9	<1·7	33·5	1·0
87 Day 0	<1·0	<2·2	32·9	2·9
85 Day 7	<1·4	<0·9	38·7	2·3
87 Day 7	<0·9	<0·9	41·9	3·1

The residual titres are expressed as the p.c. of the K value of the absorbed sera as compared with that of the sera absorbed only with YS.

TABLE 5.

Absorption of sera which had a T4 primary stimulation.

Sera	Secondary Stimulus	Absorbed with T2YS		Absorbed with T4YS	
		Residual T2	Residual T4	Residual T2	Residual T4
1145	T2	6·1	30·0	<2·6	1·0
1165	T2	2·0	35·2	<2·8	1·2
1164	T4	4·0	46·6	<8·9	1·3
1166	T4	3·0	62·6	<6·1	0·8

The residual titres are expressed as the p.c. of the K^{45} value of the absorbed sera as compared with that of the sera after absorption with YS.

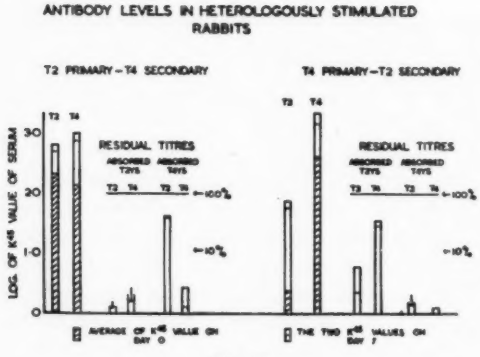

Fig. 5.

In view of the very low anti-T2 titres of the Day 0 sera from rabbits primarily immunized with T4 only the 7-day sera were absorbed. The results (Table 5) are essentially the same as in Table 4. The homologous antigen removed the great bulk of the antibody against both phages irrespective of whether the secondary stimulus was homologous or heterologous. T2, the heterologous antigen, reduced its own titre to a very low level but left specific anti-T4 in relatively large amount though as would be expected more specific antibody was left in the sera from rabbits receiving the homologous secondary stimulus.

Fig. 5 may serve to provide a summary of the essential findings with the heterologously stimulated animals in these experiments.

DISCUSSION.

In any situation where crossed serological reactions occur the first question that arises is whether we are dealing with two complexes with one or more shared antigens or whether the antigens are each single and distinct but produce antibody molecules with crossed reactivity. It is now accepted that there are at least two and possibly three different surface antigens on the phage (Lanni and Lanni, 1953; De Mars *et al.*, 1953), but that only one is concerned in "neutralization" by immune sera. This appears to be situated on the tail filaments and it is generally thought that the corresponding antibody combines with a crucial point in this area and inhibits irreversible binding to a bacterium though reversible binding is unaffected (Tolmach, 1957). However, in confirmation of Burnet (1933) and Tolmach (1957) the present results show that neutralizing antibodies can be absorbed out of antiphage sera by phage firmly adsorbed to the bacterial cell wall. This suggests that considerable areas of tail filament or adjacent phage surface, which are not essential for infective union, have the same antigenic structure as the areas which are vital for infection.

The conclusion that each phage has a characteristic single tail antigen does not, however, rule out the possibility that related antigens show cross reactions because they possess antigenic determinants in common. An antigenic determinant is always much smaller than an antigenic macromolecule and there is nothing to exclude the possibility of several different antigenic determinants being carried by a single molecule.

Jerne and Skovsted (1953) working with T4 and Cann and Clark (1954) with T2 have shown that neutralization of these phages follows a first order reaction implying that the limiting step is the union of one phage particle with one antibody molecule. Fodor and Adams (1954) using crosses between T5 and PB and Streisinger (1956) with T2 and T4 have both shown the presence of antigenic hybrids. We can assume, therefore, that there are at least two 'vital' points on the phage tail of such hybrids where union with antibody will cause neutralization. Jerne and Avegno (1956) have calculated that up to fifty antibody molecules can attach to the tail without involving a critical area.

The best working hypothesis is probably to regard neutralization as the result of union of an antibody molecule with any one of a number of critical sites on the tail antigenic area. The rate of reaction will then depend on the equivalent concentration of all antibody molecules capable of reacting with such sites.

The cross-reactions between T2, T4 and T6 have been well studied (Delbruck, 1946). Anti-T2 serum if absorbed with T4 has no titre to T4 and T6 though there is still a considerable anti-T2 remaining. Similarly, if it is absorbed with T6. This led to the idea of group and type specific antigens in this group.

Whenever absorption of serum *a* by phage B leaves reactivity of the absorbed sera against phage A we must assume that there are antibody molecules,

(1) which can react with both A and B, i.e. with some common component γ say, and (2) antibody molecules which react with A and not B indicating A has a component α not present in B. The minimum requirement in terms of an hypothesis which ascribed cross reaction to common antigenic determinants then can be represented as follows:

$$A.-\alpha\,\gamma \qquad\qquad B.-\beta\,\gamma.$$

Alternatively, we can regard the situation as involving two related antigenic determinants A and B or better A and A′. The antibody population consists then of *a* antibody specific for A and *a′* reacting with both A and A′ (B). Production of *a′* will be stimulated by A′ (B) but all the antibody produced in secondary fashion will still be absorbed by A. There might be a minor decrease in the proportion of specific antibody as compared with what results from an homologous secondary stimulus (see Table 5).

In the present system we have results essentially analogous to those obtained by Jensen *et al.* (1956) using ferrets serially immunized with influenza A strains. There is no evidence of an increase in specific homologous antibody as a result of a secondary stimulus with the heterologous antigen. What has happened is that there is a marked increase in the same type of cross-reacting antibody as was produced by primary immunization.

Such a result is not crucial in its bearing on rival theories of antibody production, but it is very easily interpreted on the clonal selection theory (Burnet, 1959). Amongst the clones stimulated by the primary immunization are *a* and *a′* of which *a′* cells can be stimulated either by A or B antigen, *a* only by A. Clones of *b* character will not be present in more than accidental numbers in the A immunized animals and the heterologous stimulus will be merely a primary stimulus as far as such clones are concerned.

Acknowledgments. I am deeply indebted to Sir Macfarlane Burnet, O.M., F.R.S., for suggesting this investigation and for his help in presenting the findings.

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TOXOPLASMA IN QUEENSLAND

III. A PRELIMINARY SURVEY OF ANIMAL HOSTS

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SUMMARY.

The *Toxoplasma* complement fixation test was used to survey 17 species of wild animals and seven species of domestic animals for reservoirs of infection.

Antibodies to a high titre were detected in two species of bandicoots, six species of rats, and the brush-tail possum. Water-rats (*Hydromys chrysogaster*) from two different areas gave the highest incidence of positive titres recorded.

There is little serological evidence of infection in animals which are consumed by man.

All of the positive sera were from the coastal strip east of the Great Dividing Range.

Toxoplasma was isolated from the bandicoot, the brush-tail possum, the marsupial rat, the climbing rat, the water-rat, the cat and the crow.

INTRODUCTION.

In an earlier paper describing the rôle of the bandicoot and rat as reservoirs of *Toxoplasma*, Pope *et al.* (1957) observed a correlation between the presence of complement fixing antibodies and the isolation of the organism, and concluded that the complement fixation test was a reliable method of detecting chronic infections in these animals. The results of a search for reservoirs of *Toxoplasma* in wild and domestic animals by means of the complement fixation test and mouse inoculation are recorded in the present paper. A serological survey of the human population is being undertaken, and will be published at a later date.

METHODS.

Complement fixing antigen, complement, and sheep blood were prepared by the methods described in the earlier paper referred to above. Antigens were stored frozen at — 20° C., without any diminution of titre over a period of five months. The RH strain of *Toxoplasma* was used, so that the results could be compared with surveys in other countries.

Complement fixation tests were performed by the method of Sabin (1949), inactivating the sera at 60° C. for 20 minutes. Swine sera, which are rich in the C_3 component of complement, received the specialized treatment of Boulanger (1955), before they were inactivated and tested in the normal manner. Dog sera, normally anticomplementary, were tested by inactivating at 56° C. for 30 minutes, and using dog complement in the test (Larin *et al.*, 1957).

Cathie (1955) stated that he did not know precisely how to evaluate a titre of 1:4, and suggested that complement fixing titres should be at least 1:8 before any significance is attached to them. For this reason, together with the fact that only small amounts of serum were available from some species, the lowest dilution of serum tested was 1:8. All titres are expressed as initial serum dilutions.

It will be noted in the result that many sera were anticomplementary, particularly from marsupials. All efforts to remove the anticomplementary factor by the recognised methods for human sera (Taran, 1946; Rapp *et al.*, 1955; Ruge, 1956) failed, and, as these sera cannot be considered to have been effectively tested, they are excluded whenever the percentage of positive sera for a given species is expressed.

The sera (with three exceptions) were not tested by the *Toxoplasma* dye test of Sabin and Feldman (1948), as it has been suggested by Cathie (1957) that sera of domestic and wild animals contain a toxoplasm-hostile factor, which produces low titres in the dye test. Added to this, the accessory factor needed for the test has been difficult to obtain, with the result that the number of dye tests performed has been reduced to a minimum.

Techniques used in the mouse inoculation work were essentially those described previously (Pope *et al.*, 1957). Three passages were made in mice before dismissing an attempt at isolation as negative. The strains isolated were identified as *Toxoplasma*, on the basis of transmissibility in mice, with production of typical splenomegaly and pneumonitis, and presence of pseudocysts in the brain.

MATERIALS.

Sera investigated.

Sera have been tested from 497 wild mammals, representing 17 species (including the small series reported by Pope *et al.*, 1957), and from 428 domestic mammals of seven species. Details are shown in Tables 1 and 2. Most were collected in the course of other investigations by the Institute, particularly at the Innisfail Field Station in north Queensland; but specimens from many of the domestic and some of the wild animals from south Queensland were received through the kindness of the Royal Queensland Society for the Prevention of Cruelty, the Brisbane City Council, and the Queensland Department of Agriculture and Stock.

Three geographical regions are represented: south-eastern Queensland (about 27° S.), subtropical sclerophyll woodland, with patches of rain-forest, and a predominantly summer rainfall averaging 40 in. per annum; the wet-tropical, coastal belt from Mossman to Innisfail (16-17° S.), largely covered by rain-forest, and with an annual rainfall of 80 to 140 in.; and the drier, western country from Tambo to Roma (25-27° S.), comprising open plains, dry scrubs and patches of sclerophyll woodland, and with a relatively unreliable rainfall of about 20 in. per annum. Human settlement is dominant in the south, important in the north, and sparse in the west. A few specimens came from other areas (1 *Macrotis* from Birdsville, 7 sheep from Hughenden, 19 cattle from Charters Towers), those received from the three defined regions being: Southern: 44 bandicoots, 8 possums, 130 rodents, 29 cats, 30 dogs, 30 horses, 7 goats, 49 sheep, 34 cattle, 11 pigs. Northern: 87 bandicoots, 168 rodents, 10 bats, 38 cattle, 11 pigs. Western: 49 kangaroos, 11 horses, 33 goats, 101 sheep, 18 cattle.

Autopsy and mouse inoculation studies.

Autopsies and mouse inoculation were carried out on 163 animals (Table 3), most of which were collected in the south-eastern and northern districts, while the 27 *Rattus villosissimus* were collected at Richmond in north-west Queensland. The animals studied, including those from which *Toxoplasma* were isolated, appeared healthy at the time of autopsy, with the exception of the marsupial rat, the crow and two young *T. obesulus*, which were dead on examination.

RESULTS.

Serology of wild mammals.

The results of the complement fixation tests on these sera appear in Table 1. In the marsupials, there was evidence of a substantial incidence of infection in both species of bandicoot and in the possum, but none in the kangaroos. Five swamp wallabies (*Wallabia bicolor*) were also tested, but their sera were anti-complementary and are not included in the Table. Among the rodents, serum antibodies were detected in the six species of which adequate numbers were examined, the highest proportion of positives (about 90 p.c.) and the highest

TABLE 1.

Toxoplasma *C.F. survey of wild animals.*

Species	No. tested	Anti-comp. or non-spec.	Neg.	No. positive at titres				Total pos.	p.c. pos.
				1:8	1:16	1:32	1:64 or more		
MARSUPIALS									
Thylacis obesulus (Short-nosed bandicoot)	124	35	54	2	6	8	19	35	39
Perameles nasuta (Long-nosed bandicoot)	7	—	5	—	—	—	2	2	29
Macrotis lagotis (Rabbit bandicoot)	1	—	1	—	—	—	—	—	—
Trichosurus vulpecula (Brush-tail possum)	8	1	6	—	—	—	1	1	14
Macropus spp.* (Grey and red kangaroos)	49	18	31	—	—	—	—	—	—
RODENTS									
Rattus rattus (Black rat)	131	10	117	1	1	—	2	4	3
R. norvegicus (Brown rat)	50	6	41	1	1	1	—	3	7
R. assimilis (Allied rat)	47	14	30	—	1	—	2	3	9
R. conatus (Field rat)	13	1	11	—	—	1	—	1	8
Hydromys chrysogaster (Water-rat)	25	2	2	1	2	2	16	21	91
Uromys caudimaculatus	5	—	5	—	—	—	—	—	—
Melomys spp.+ (Climbing rats)	27	4	21	—	1	—	1	2	9
BATS									
Pteropus conspicillatus (Flying fox)	8	—	8	—	—	—	—	—	—
Rhinolophus megaphyllus (Horseshoe bat)	1	—	1	—	—	—	—	—	—
Hipposideros bicolor albanensis (Brown horseshoe bat)	1	—	1	—	—	—	—	—	—

* 42 *M. major*, 7 *M. rufus*.
+23 *M. littoralis*, 4 *M. cervinipes*.

titres (70 p.c. were 1:64 or greater) being in the water-rats. No antibodies were detected in the sera of the ten bats tested.

Serology of domestic mammals.

In the domestic animals (Table 2), antibodies were detected only in two dogs and one horse from south Queensland, and in one cow from north Queensland. Except for one dog, the titres were low, and their significance is doubtful.

TABLE 2.

Toxoplasma *C.F. survey of domestic animals.*

Species	No. tested	Anti-comp. or Non-spec.	Neg.	No. positive at titres				Total pos.	p.c. pos.
				1:8	1:16	1:32	1:64 or more		
Cats	29	—	29	—	—	—	—	—	—
Dogs	30	1	27	1	1	—	—	2	7
Horses	41	—	40	1	—	—	—	1	2
Goats	40	—	40	—	—	—	—	—	—
Sheep	157	1	156	—	—	—	—	—	—
Cattle	109	27	81	1	—	—	—	1	1
Pigs	22	4	18	—	—	—	—	—	—

TABLE 3.

Isolation of Toxoplasma *from animals by mouse inoculation.*

Animal	No. examined	Isolations of *Toxoplasma*	Locality
MARSUPIALS			
Thylacis obesulus	10	5	Brisbane, Innisfail
Trichosurus vulpecula	7	2	Brisbane
*Phascogale tapoatafa**	1	1	Burleigh
Macrotis lagotis	1	0	Birdsville
RODENTS			
Rattus rattus	16	0	Innisfail
R. conatus	12	0	Innisfail
R. villosissimus	27	0	N.W. Qld.
Mus musculus	26	0	Innisfail
Melomys littoralis	14	0	Innisfail
M. cervinipes	11	1	Innisfail
Hydromys chrysogaster	4	3	Innisfail
	3	2	Brisbane
DOMESTIC			
Cats	30	1	Brisbane
BIRDS			
Corvus coronoides	1	1	Brisbane
	163	16	

* Marsupial rat.

Isolations from wild and domestic animals.

Toxoplasma was isolated from 16 animals (Table 3). The strains were uniformly of low virulence for mice, which suffered only the inapparent type of infection previously described (Pope *et al.*, 1957). Pseudocysts were observed in sections of brain of both the infected water-rats from Brisbane and one infected possum. Small pseudocysts were found in the heart of the other infected possum. Examination of the tissues of the marsupial rat revealed severe myocarditis, with associated proliferative forms of *Toxoplasma*, and it may be assumed that the animal died of toxoplasmosis. No toxoplasms nor histopathological signs of *Toxoplasma* infection were demonstrated in the sections of tissues of the crow or of the cat from which isolations were made. As no complement fixing antibodies were detected in the cat's serum, it appears that the infection was probably in an early stage.

Natural infections in the laboratory.

A series of infections occurred in three bandicoots which were housed in the Institute's insectary. They comprised a female *T. obesulus*, captured in a Brisbane suburb on 9th August, 1957, and her two pouch young, both males, which were very small and naked when she was captured. They began to grow hair about 14th September and to leave the pouch on 23rd September, when they weighed 75 and 77 gm.

The mother appeared well until 3rd October, but was very ill on the 4th, weak and listless, lying on her side outside her box, and showing some nasal discharge. She was diagnosed as suffering from pneumonia, but recovered steadily, and was well again by the 7th. She was killed on 5th November for investigation. The autopsy showed a large abscess in the left nasal sinus just in front of the eye. The bone was decayed away above the sinus, and the head had a swollen appearance. *Toxoplasma* was isolated from her tissues. Her serum was anticomplementary in the complement fixation test, but gave a dye-test titre of 1:4096.

The infant that weighed 77 gm. on 23rd September developed steadily, and weighed 126 gm. on 9th October. On the 11th, it weighed 121 gm., when it appeared well, and it weighed 100 gm. and was obviously very sick on the morning of the 14th. Later in the day, it was seen to have a fit, blood-stained fluid ran from its nose, and it died a few minutes later.

The second infant also grew steadily, reaching 189 gm. on 15th October. Its weight was practically stationary at 187-186 gm. from the 16th to the 18th, during which period it did not appear ill, but it was found dead at 4.30 p.m. on the 18th.

Both the young bandicoots showed severe oedematous pneumonitis at autopsy, and numerous free toxoplasms were present in lung smears stained with Giemsa. *Toxoplasma* was isolated from the tissues of both, and also from

washings of the nasal sinus collected four hours after death from the one that had an obvious nasal discharge. The sera of both were negative to the complement fixation test, but the second to die gave a dye-test titre of 1:256, the other being negative.

This group of infections is of unusual interest from two points of view. In the first place, it shows that strains of *Toxoplasma* occurring in Australia are, at least sometimes, highly pathogenic to bandicoots by what must be presumed to be natural routes of infection. The findings in the marsupial rat indicate that they may be pathogenic to other marsupials also. Nevertheless, the kind of serological results reported here from animals collected in the field would be possible only if the general recovery rate were high.

Secondly, it seems almost certain that the infants, and probably their mother too, were infected in the insectary. This would exclude insect transmission, because there were no free insects in the insectary to infect them. The actual source of infection is obscure. It seems evident that the mother was infected first, and her infants from her, or possibly one infant from her and the other from it. Other bandicoots and native rats were housed in the insectary during the relevant period, and infection may have come from them. It is even possible, though unlikely, that the mother was already infected when she was captured. The infectivity of the nasal secretions may be significant, because infected droplets would provide an adequate pathway of infection in this enclosure, where bandicoots were allowed to roam freely about the floor from time to time.

DISCUSSION.

The most striking feature of the results is the contrast between the wild and domestic animals. Evidence of infection has been found in dogs, cats and sheep by Wickham and Carne (1950), and *Toxoplasma* has been isolated from one of the cats in the present series. Nevertheless, if the serology is to be trusted,[1] the incidence of infection in domestic animals is much lower than the overall 14 p.c. so far indicated for the human population of Queensland. It might be inferred, therefore, that, while the household pets may sometimes be a link in the chain of infection leading from wild animal to man, eating imperfectly cooked meat is unlikely to have the significance that Skieller and Smetana (1958) were inclined to attribute to it in India.

There is a contrast within the wild animal series also, in that infection appears to be much more frequent in the native small ground mammals of the bush than in the introduced brown and black rats of the towns and canefields (33½ p.c. of 191 effectively tested, as against 4 p.c. of 165). Again, the figures

[1] It cannot yet be accepted as confidently for the domestic animals as for the bandicoots and rodents.

must be accepted with caution until more balanced data are available; but they do suggest that the strains of *Toxoplasma* encountered may be native to this country, long adapted to live in association with our native fauna, and that the introduced rats, as well as human beings and domestic stock, may be no more than secondary hosts.

Among the native animals, the results reported here confirm the earlier finding (Pope *et al.*, 1957) that bandicoots are important reservoirs of *Toxoplasma*, and extend it to include substantial areas of country in two widely separated parts of coastal Queensland. They have also added several new hosts, among which the water-rats, *Hydromys chrysogaster*, stand out as possibly even more efficient than the bandicoots.

Biological efficiency (as indicated by unimpaired activity of the host, long duration of infection, and free exchange of parasites, all of which contribute to a high incidence of infection detectable in surveys of this kind) is not the only factor that determines the significance of a host in the epidemiology of human infection; abundance and association with the human population are at least equally important. From these latter points of view, the bandicoots are likely to be more important than the water-rats, and the domestic rats, in spite of the relatively low incidence of infection in them, more important than either. It is difficult to define chains of infection without knowing the pathway and method of infection, but something like the following sequence of events may be visualized.

Water-rats are slow breeding, aquatic, and fairly common in the coastal creeks, where they build a system of burrows in the creek banks. Their diet includes fresh water fish, molluscs, yabbies, young aquatic birds and their eggs, and some vegetable material (McNally, 1958). They do not have direct contact with humans, except perhaps through contamination of water supplies, but they do have contact with bandicoots, possums, native rats and *R. rattus*, all of which are proven hosts. The native rats would merely serve to enlarge the general reservoir of infection in the environment, but the other species could carry it into contact with the human population.

Bandicoots are widespread and numerous in the coastal areas of Queensland, being found in the high grass surrounding creeks and swamps. They are commonly present in suburban gardens, and their diet is mainly carnivorous, including worms, slugs, snails, insects, and their larvae (Sandars, 1950). Possums often make their homes in the ceilings of houses, ranging out at night to feed on eucalypts in the gardens and adjacent bush, while *R. rattus* encounters the more strictly urban *R. norvegicus* in the same general environment. The rats could carry infection directly into the houses, while dogs and cats could contribute through their contacts with both the rats and the marsupials.

A chain of this kind is an attractive hypothesis, but much more work is needed before its validity can be established. This applies particularly to the

method, or methods, of transmission in nature, about which we can as yet say little, apart from the suggestion of droplet infection in the two young bandicoots that became infected in the laboratory.

In the geographical analysis, an interesting observation is the absence of any evidence of infection in the 239 animals from places with a rainfall less than 25 inches. This supports the suggestion by Jacobs (1957) that toxoplasmosis is more prevalent in moist, warm areas than in hot or cold, dry areas. There were no inland samples from species that are known to be good hosts on the coast, so a satisfactory comparison cannot be made; but the failure to recover toxoplasms from 27 *Rattus villosissimus* from Richmond, and the known susceptibility of macropids mentioned by Pope *et al.* (1957), would support the opinion that *Toxoplasma* is at least relatively uncommon west of the Dividing Range. On the other hand, although there is some variation between species, there is no overall difference between the results that can be compared from north and south coastal Queensland.

Finally, the correlation of the results of mouse inoculation and the complement fixation test provides further evidence of the reliability of complement fixation for testing marsupial and rodent sera (*cf.* Pope *et al.*, 1957, Table 2). Sixty-four animals were tested by both methods, the organism being isolated from 12. The sera of two of these were anticomplementary, seven gave strong complement fixing titres, and the remaining three were negative. The three negative sera were from the two young bandicoots, which obviously died in the acute stage of infection, and the cat, which was presumably also in an early stage. The sera of 49 of the 52 animals negative by inoculation were negative to the complement fixation test, two were anticomplementary, and the remaining one, from a water-rat, was positive (titre 1:64). This is the first indication that infection may not persist throughout the lifetime of the animal. The chief disadvantage of the complement fixation test would seem to be the prevalence of the anticomplementary factor in marsupials. It is unfortunate that a similar correlation cannot yet be attempted with material from domestic animals.

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THE ISOLATION OF ORPHAN VIRUSES FROM PIGS IN NEW ZEALAND

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SUMMARY.

Five cytopathogenic agents have been isolated from pig faeces, and their characteristics suggest that they are viruses. They are serologically identical, are non-pathogenic for piglets or laboratory animals, and will not produce infection in chick embryos, HeLa, bovine or dog epithelial tissue cultures. Neutralizing antibodies against the viruses are widespread in apparently healthy pigs, and very young piglets had evidence of maternal antibodies. On one occasion the virus was recovered from an experimentally inoculated animal by spontaneous degeneration of tissue cultures prepared from the kidneys of that animal. The virus produces a complement fixing antigen in tissue culture, which can be used to detect antibodies in pig serum by the indirect complement fixation test but not by the direct complement fixation test.

INTRODUCTION.

During the routine examination of pig faeces in pig kidney monolayer tissue cultures several cytopathogenic agents were isolated. It is the purpose of this paper to deal with the characteristics of these agents in tissue culture, their distribution in healthy pigs, and their pathogenicity for suckling pigs.

MATERIAL AND METHODS.

(a) Three apparently healthy 2-3 months old pigs were received from a farm with a history of sporadic deaths. The pigs were normal on clinical, bacteriological and parasitological grounds. A separate faeces sample was obtained from each pig.

(b) Two pigs 2-3 months old were received from a farm with a history of ill-thrift and sporadic deaths. The pigs were infected with mange and lungworms, but showed no evidence of a virus infection on clinical or histological grounds. Faecal and serum samples were obtained from one pig, and faecal, serum, lung and pericardial samples were obtained from the second pig.

Tissue culture system.

Tissue cultures of pig kidney were prepared by the method of Bodian (1956) and the primary culture was grown in the Lactalbumin hydrolysate medium of Cheatham and Crandell (1957). The maintenance medium contained only 5 p.c. of horse serum.

Antisera.

Hyperimmune sera were prepared in rabbits inoculated with multiple doses of tissue culture fluid with a T.C. of 10^6.

Neutralization test.

Neutralization tests were carried out using 100 T.C. I.D. 50 of the agent and dilutions of serum. The test was read 24-28 hours after the control tubes showed complete lysis.

Complement fixation.

Direct complement fixation tests were carried out by the method of Webster (1958) and indirect by that of Karrer *et al.* (1950) using undiluted tissue culture fluids as antigen.

Piglets used for transmission experiments.

Two groups of three piglets were used in transmission experiments. The first group was obtained at five days of age. The second group was born directly into sterile dishes and washed in disinfectant. They did not have access to colostrum. Both groups were reared under artificial conditions on a diet containing 10 units of both penicillin and streptomycin per ml. of food. The diet consisted of tinned concentrated cow's milk 49 p.c., water 49 p.c., essential minerals 1 p.c. and cod liver oil 1 p.c.

RESULTS.

Isolation of agents in tissue culture.

Large numbers of highly refractile cells and small areas of cellular degeneration were observed 24-48 hours after infection of cultures with faeces. The areas of cellular degeneration increased rapidly, and 24 hours later the whole cell sheet was lysed. No agents were isolated from the organs of the animal submitted for examination.

Each agent was passaged six times in tissue culture so that the final dilution of the initial inoculum was over 10^{-15}. Each isolate was examined aerobically and anaerobically for bacteria. P.P.L.O. investigations in enrichment cultures, agar plates and chick embryos, showed that the agents did not fall into these groups. The growth characteristics of the agents in tissue culture, their filterability, and their ability to be neutralized with antisera suggested that the agents were viruses and they will be referred to as such for the rest of this paper.

Growth characteristics.

Fig. 1 shows the rate of release of the virus from infected cells. There are four definite phases: up to 12 hours no free virus could be detected in the cultures; then a phase of gradual increase

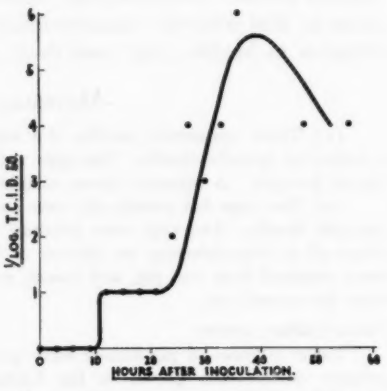
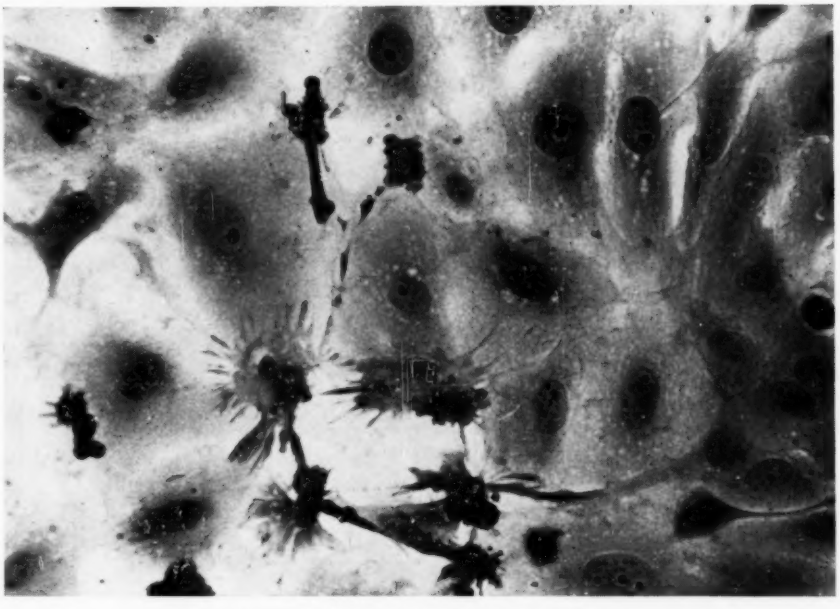


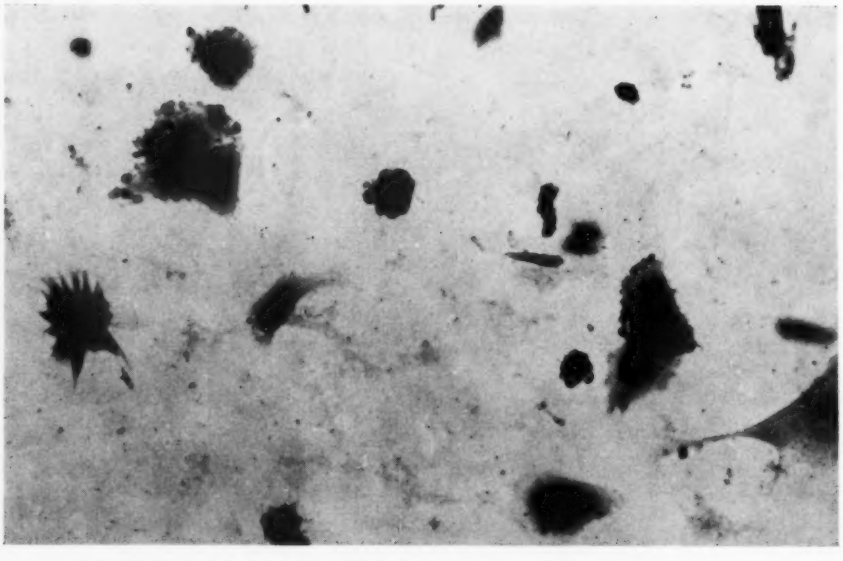
Fig. 1. Rate of release of pig orphan virus from pig kidney tissue cultures.

Fig. 2. Cytopathogenic changes produced in pig kidney tissue culture by pig orphan virus. (a) Normal cultures prior to infection.



(b) Infected cells 24 hours after inoculation.

(c) Infected cells 36 hours after inoculation.



(d) Infected cells 48 hours after inoculation.

followed by a phase of rapid release and finally a gradual fall from the maximum virus concentration. Histological studies showed that highly refractile cells began to appear 12 hours after infection and increased in number up to 24 hours when definite plaques of degeneration could be detected. The areas of cellular degeneration were characterised by stellate cytoplasmic processes from disrupted cells, a dense nucleus, and a moderate amount of multinucleation in cells near the sites of degeneration. By the 36th hour post-inoculation only isolated islands of cells remained and by the 48th hour the cells were completely lysed (Fig. 2 a, b, c, d).

Estimation of size of the virus particle.

Preliminary filtration experiments showed that the virus would pass through "Gradacol" membranes with an average pore diameter of 66 mμ but not through membranes with an average pore diameter of 27 mμ. Prefiltration of the virus suspension through membranes with an average pore diameter of 290 mμ resulted in the virus passing through membranes with an average pore diameter of 50 mμ but not through membranes with an average pore diameter of 27 mμ.

Host range.

The reference virus with a T.C.I.D.$_{50}$ of 10^6 was inoculated by the intraperitoneal, intracerebral, and intravenous routes into laboratory animals. The virus was not pathogenic for rabbits, rats, guinea-pigs or mice. Three blind passages in suckling, weaner and adult mice gave no significant results.

Blind passage of the virus in HeLa cells, dog kidney epithelial cells and bovine kidney epithelial cells gave no cytopathogenic changes and the virus could not be isolated from the first passage in any system.

All attempts at passaging the virus in the chick embryo were negative. The virus could not be isolated after three blind passages on the chorio-allantoic membrane, in the yolk sac or in the allantoic cavity.

Transmission to suckling pigs.

Two attempts were made to infect piglets with virus grown in tissue culture. The first group of three piglets was found to have low titres of maternal antibody, so the second group of three piglets was obtained before access to colostrum.

Various routes of inoculation were tried, including intracerebral, but the piglets showed no disease symptoms. In every case the virus was isolated from the faeces for periods up to one month after infection and all piglets showed a marked rise in neutralizing antibody titres. Monolayer kidney cultures prepared from one piglet resulted in the spontaneous degeneration of the cultures and the isolation of the virus.

Serological properties of the virus.

All examinations of the virus for the presence of a haemagglutinin were negative. Tests were carried out over the pH range from 5·7 to 9·1 at 4° C., 18° C. and 37° C. for short periods and overnight. The use of sheep, fowl, one-day chick or pig red blood cells did not influence the results.

Direct and indirect complement fixation tests were carried out on pig sera. Only the indirect test was positive. There was a clear correlation between the titre by the indirect C.F. test and by the neutralization test. Rabbit antisera gave high titres in the direct complement fixation and neutralization tests.

A limited number of pig sera was obtained from the local abattoir during the investigation. The 56 serum samples were collected at random during two days when approximately 500 pigs were killed. The pigs were from six months to one year of age and were apparently healthy and in excellent condition. The results of neutralization tests on these sera are recorded in Table 1.

The results in Table 1 suggest that most pigs have at some stage been in contact with the virus and suggest that the virus is probably widespread in the pig population of New Zealand.

TABLE 1.

Neutralizing antibody titres of pig sera collected from the local abattoir.

Number of sera	Number of animals with serum-neutralization titres of:				
	<1/5	1/5	1/10	1/50	>1/50
56	12	3	12	4	25

In 1956 Moscovici *et al.* reported the isolation of a virus from the faeces of pigs suffering from enteritis. The virus was neutralized by ECHO 8 antisera but not by other ECHO antisera or poliomyelitis antisera. As the virus under discussion has most of the properties described by Moscovici *et al.* (1956) it was decided to carry out neutralization tests with readily obtainable ECHO and poliomyelitis antisera. Neutralization tests were carried out with Polio Type I, Polio Type II, Polio Type III, ECHO types 6, 6[1] (V186), 9 and 8 antisera. The virus was not neutralized by any of the ECHO antisera tested or with Polio Type I or II; but a small degree of neutralization was obtained with Polio Type III antiserum. 10 $T.C.I.D._{50}$ of the virus were neutralized by Polio Type III antiserum, but 100, or 1,000 $T.C.I.D._{50}$ were not neutralized. The reverse neutralization did not occur; Polio viruses Types I, II or III were not neutralized by the pig virus antiserum.

Cross neutralization tests with the five viruses isolated showed that they were serologically identical.

DISCUSSION.

From the experimental studies it would appear that the viruses isolated were not the cause of sporadic deaths on the farms from which the samples were obtained.

The experimental results obtained with the viruses under discussion appear to be very similar to those obtained by Beran *et al.* (1958) with the entero viruses of swine. Serological cross reactions have not yet been carried out, but these are planned when sera are obtained.

Although the results suggest that this virus is non-pathogenic, the transmission experiments do not rule out the possibility that the virus may be pathogenic under some conditions. The results do, however, exclude the possibility that this virus is Talfan or Teschen Disease. The direct complement fixation test with hyperimmune rabbit serum will be of further value for rapid typing of further virus isolates from pigs.

Hsiung and Melnick (1958) applied the name ECSO (enteric cytopathogenic swine orphan) virus to the apparently non-pathogenic pig viruses, and it would appear that the five viruses isolated could be included in this group. In a recent review of the orphan viruses of man and animals, Hsiung and Melnick (1958) showed that orphan viruses have been isolated from man, monkeys, mice, cattle and pigs and it seems probable that they will be isolated from all animals. At least eight of the fourteen antigenically distinct human orphan viruses have been associated with cases of aseptic meningitis so that it is possible that some of the other animal orphan viruses will be associated with disease.

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A STUDY OF THE HORMONE CONTENTS OF THE ROSTRAL AND CAUDAL ZONES OF THE PARS ANTERIOR OF THE WALLABY PITUITARY

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SUMMARY.

Hormone assays of the rostral and caudal zone of the pars anterior of the wallaby pituitary showed a high concentration of prolactin and thyrotrophin in the rostral zone, and a high concentration of somatotrophin in the caudal zone.

Gonadotrophin and corticotrophin were distributed throughout the whole gland.

The distribution found shows that prolactin and somatotrophin are formed by different cells. Correlation with observable cell types suggests that prolactin may be formed by an acidophil cell with an affinity for acid fuchsin, and somatotrophin by an acidophil cell with an affinity for orange G. These identifications require confirmation from functional studies.

INTRODUCTION.

In a previous paper from this department on the cytology of the pars anterior of the wallaby pituitary (Ortman and Griesbach, 1959), two types of acidophils distinguished by the presence of differently staining granules are described. In one type of cell these granules are stained red by acid fuchsin and in the other they are stained orange by orange G. The red or fuchsinophil cell is restricted to the rostral zone and the orangeophils are distributed generally throughout the gland. It is possible by means of a single cut in the correct plane to separate the pars anterior into rostral and caudal zones containing predominantly fuchsinophils and orangeophils respectively.

A study of the hormone concentration in the rostral and caudal zones is reported here. Thyrotrophin, prolactin, somatotrophin, gonadotrophin, and corticotrophin responses have been investigated by conventional methods.

MATERIALS AND METHODS.

Pituitaries were taken from freshly killed scrub wallaby (*Wallabia rufogrisea*) shot in the Hunter Hills, Waimate, New Zealand. The pituitaries after removal were placed immediately in dry acetone, and later stored in dry acetone at — 20° C. While still wet with acetone the dura and posterior lobe were removed and the pars anterior cut into two parts following the instructions of Ortman and Griesbach (1959). After removal of the surplus acetone under reduced pressure the material was ground to a fine powder and stored in the dry state at a low temperature.

Thyrotrophin assay. 50 mg. of acetone-dried female pars anterior from each zone was extracted with acetone HCl to remove the prolactin (Lyons, 1936). The residue was washed with acetone, dried, and then extracted for 1 hour at room temperature with a mixture containing 1 ml. 0·1N NaOH and 2 ml. 0·1M Na_2HPO_4 and then centrifuged. The supernatant was removed and the residue extracted for 1 hour with 0·5 0·1M Na_2HPO_4. The combined supernatants were made up to 5 ml. with 0·1M KH_2PO_4 and the final pH of the extract was 7·0.

To provide a suitable potency for the thyrotrophin assay, the extract was diluted with saline. With both the rostral and caudal extracts the amount used for each injection was equivalent to 100 μg. of the acetone-dried starting material.

Thyrotrophin was assayed by the method of Adams and Purves (1955 and 1957). This method depends on the labelling with I^{131} of the iodine compounds in the thyroid of a guinea-pig, and the inhibition of secretion of thyrotrophin by the animal's own pituitary by the administration of adequate amounts of thyroxine. In animals so treated the rise in radioactivity of the blood following injection of thyrotrophin is a measure of the amount of thyrotrophin injected.

Prolactin assay. Acetone-dried rostral and caudal powders from male wallaby pars anterior were suspended in phosphate saline at pH7 and assayed by the local crop sac stimulation method of Bates (1950), using squab pigeons. The rostral suspension was injected on one side of the crop, and the caudal suspension at the same concentration on the other side, so that the responses to the two zones could be compared in each bird. The total dose was either 0·4 mg. or 1·6 mg., which was given in 0·1 ml. intradermal injections for four days, and the birds killed on the fifth day. The dissected crop sacs were examined under tension with transmitted light for a local response, any difference between the two sides noted, and the response on each side rated on an arbitrary scale 0-5.

Assays in hypophysectomised immature rats. Somatotrophin, gonadotrophin and corticotrophin were assayed in hypophysectomised immature male and female albino rats from the local colony. These were used 8-15 days after operation. 2 mg. per day of acetone-dried pituitary powder as a suspension in 0·5 ml. of phosphate saline at pH7 was injected subcutaneously, and the rats were weighed daily.

In the first experiment powders prepared from the rostral and caudal zones of pooled wallaby pituitaries were compared, the injections being continued for 14 days. In the second experiment powders prepared from the rostral and caudal zones of male wallaby pituitaries were compared, the injections being continued for 11 days.

At autopsy, the gonads, accessory organs and adrenals were removed, freed from fat, weighed and compared with those from uninjected colony controls.

The sella turcica of all the rats included in the results was checked microscopically to ensure that no pituitary remnant was present. The ovaries, testes, and the ventral prostates from the second experiment were examined histologically.

RESULTS.

Thyrotrophin. A positive response was given by the extract of the rostral zone and no TSH was detected in the caudal zone. Fiducial limits at $P = 0·05$ are given for the response from the rostral zone.

Rostral Zone:	Found 0·01 I.U. TSH/mg.
Limits ($P = 0·05$)	$\begin{cases} < 0·016 \text{ I.U. TSH/mg.} \\ > 0·004 \text{ I.U. TSH/mg.} \end{cases}$
Caudal Zone:	< 0·002 I.U. TSH/mg.

Prolactin. The side of the crop sac which received the rostral suspension was always more stimulated than that which received the caudal suspension. At the 0·4 mg. dose level the caudal suspension failed to give a response in 4 out of 7 birds, and the mean difference in rating in 7 birds was 1·14 ± 0·4 S.E.M. At the 1·6 mg. dose level both sides were stimulated. The mean difference in the rating of the responses from the rostral and caudal zones in 7 birds was 1·43 ± 0·42 S.E.M. (Table 1).

TABLE 1.

A comparison of the prolactin content of the rostral and caudal zones of male wallaby pars anterior at 0·4 mg. and 1·6 mg. dose levels.

0·4 mg.				1·6 mg.			
Rating of Crop Sac Response				Rating of Crop Sac Response			
No.	Rostral	Caudal	Difference (Rostral-Caudal)	No.	Rostral	Caudal	Difference (Rostral-Caudal)
1	2	1	1	1	3	2	1
2	1	0	1	2	3	1	2
3	1	0	1	3	2	1	1
4	2	1	1	4	3	1	2
5	1	0	1	5	2	1	1
6	1	0	1	6	3	1	2
7	3	1	2	7	4	3	1
			Mean 1·14 S.E.M. ±0·4				Mean 1·43 S.E.M. ±0·42

Somatotrophin. There was no mean increase in body weight in the 6 rats receiving the rostral suspension, and the mean increase of the 6 rats receiving the caudal suspension was 17·2 gm. The difference between the two groups is highly significant $p < 0·001$ (Tables 2 and 3 and Fig. 1).

Gonadotrophin. Positive gonadotrophic responses were obtained from both zones, as shown by an increase in the weight of the gonads over controls (Table 3).

Histological examination showed that the male rats receiving the rostral suspension had immature testes and mild stimulation of the columnar epithelium of the ventral prostate. Males treated with the caudal suspension had immature testes, but there were numerous mitoses, the tubules were enlarged, and the prostatic epithelium was activated with the Golgi ring visible.

The ovaries of the female rats receiving the rostral suspension had numerous enlarged corpora lutea with few follicles; the ovaries of the rats receiving the caudal suspension were almost completely luteinised with a few inactive follicles similar to a pregnancy ovary.

TABLE 2.

Body weight changes in hypophysectomised immature rats receiving 2 mg./day of the rostral or the caudal zone of wallaby pars anterior.

Rostral Zone			Caudal Zone		
Sex	Initial Weight gm.	Weight Increase gm.	Sex	Initial Weight gm.	Weight Increase gm.
♀	74	0	♀	60	11
♀	72	3	♂	79	22
♀	70	−1	♀	87	13
♀	80	−3	♀	68	15
♂	71	0	♂	74	25
♂	73	1	♂	74	17
		MEAN 0 S.E.M. ±0·8*			MEAN 17·2 S.E.M. ±2·5*

* The difference between the means is highly significant $P<0·001$.

Corticotrophin. The adrenals from all the injected animals were increased in weight as compared with the controls, and there was no difference in the responses produced by the two zones (Table 3).

TABLE 3.

The responses of hypophysectomised immature rats to daily injections of 2 mg./day saline suspensions of wallaby pars anterior.

Experiment 1. Pooled pars anterior 2 mg./day for 14 days began 15 days post operation.

		Initial Weight gm.	Weight Increase gm.	Weight of Organs mg.					
				Ovaries	Uterus	Testes	Ventral Prostate	Seminal Vesicles	Adrenals
Rostral Zone	♀	74	0	49	83				
	♀	72	3	49	86				
Caudal Zone	♀	60	11	12	70				
	♂	79	22			734	42·2	36·6	

Experiment 2. Male pars distalis 2 mg./day for 11 days began 8 days post operation.

Rostral Zone	♀	70	− 1	67	106				14·6
	♀	80	− 3	67	158				16·6
	♂	71	0			610	11·0	12·0	12·9
	♂	73	1			601	11·2	8·7	11·4
Caudal Zone	♀	87	13	122	174				15·1
	♀	68	15	104	190				13·4
	♂	74	25			914	45·1	31·5	12·7
	♂	74	17			908	33·3	36·0	14·6
Hypophysectomy Colony Controls	♀	76	− 3	5	24				
	♂	76	− 3			180	6	5	6

DISCUSSION.

The two zones of the wallaby pars anterior, characterized by differences in cell types revealed by staining reactions, have shown clear-cut differences in hormone content. The rostral zone has higher concentrations of prolactin and thyrotrophin, and the caudal zone has a higher concentration of somatotrophin Both zones contain gonadotrophin and corticotrophin.

Prolonged injections of gonadotrophin to hypophysectomised immature rats do not favour a separate identification of follicle stimulating and luteinising hormone. The size of the ovaries indicates that follicle stimulating hormone was present, but the effect after a long series of injections was predominantly luteinising. The presence of luteinising hormone is also indicated by the stimulation of the ventral prostate. From the small amount of material available the results suggest that there is more luteinising hormone in the caudal zone.

The relation of hormone content to specific cell types.

Two kinds of acidophilic cells have been seen in the pars anterior of a large number of species; amongst these are man (Romeis, 1940); the ferret (Dawson, 1946); and the opossum (Dawson, 1938). Differential hormone assays, however, have only been reported on zones rich or poor in acidophils generally. A number of assays have been done on the acidophilic and basophilic zones of the bovine pituitary. Smith and Smith (1923) found growth hormone predominantly in the acidophilic zone and thyrotrophin predominantly in the basophilic zone.

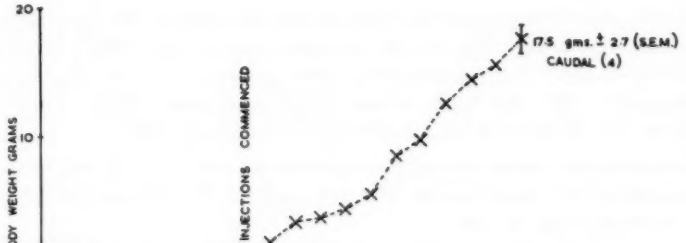

Fig. 1. *A comparison of the growth rates of hypophysectomised immature rats receiving 2 mg./day of the rostral or the caudal zone of male wallaby pars anterior.* The means of groups of four rats at the end of the experiment were rostral 0·8 gm. ± 0·9 S.E.M. and caudal 17·5 gm. ± 2·7 S.E.M.

Azimov and Altman (1938), and Friedman and Hall (1941), showed a high concentration of prolactin in the acidophilic zone, and Smelser (1944) showed a high concentration of gonadotrophin and thyrotrophin in the basophilic zone. Generally it is considered that somatotrophin and prolactin are made by acidophilic cells, and the gonadotrophins and thyrotrophin are made by basophilic cells. As yet no agreement has been reached as to the type of cell which produces corticotrophin.

The separation of two acidophilic cell types in the wallaby pars anterior has permitted the determination of the relative concentrations of prolactin and somatotrophin in the two zones containing different types of acidophils. The difference in prolactin and somatotrophin content in the two zones shows that these two hormones are produced at different sites and therefore in different cell types. The obvious inference is that the fuchsinophil secretes the prolactin, and the orangeophil the somatotrophin, as appears to be true for the two acidophilic types in the dog (Purves and Griesbach, 1957). The fact that some prolactin was found in the caudal zone is easily attributed to the inclusion of some fuchsinophils in the caudal portion, since it is not possible to control the separation by histological examination of each pituitary. The absence of any growth response from the rostral portion does not correlate so well with the distribution of orangeophil cells since these are found in both zones. Ortman and Griesbach (1959) found the orangeophils in the rostral zone were smaller than those in the caudal zone and also showed other small differences. They may, therefore, be a type of cell distinct from the large orangeophil of the caudal zone.

Of the cells distinguished by Ortman and Griesbach, the basophil with the granules staining faintly blue by the Crossman procedure (1937) corresponds in its distribution with the thyrotrophin, since it is restricted to the rostral zone. The basophils with the blue or/and purple granules which are distributed throughout the gland may be gonadotrophin secreting cells.

Corticotrophin shows, as in other species studied, a fairly uniform distribution throughout the gland which does not correspond with any particular acidophil or basophil type of cell.

Identifications of functional cell types based on correlations between morphological cell types and hormone contents can only be tentative, and require confirmation by studies of the responses of the cell types to physiological and experimental alterations of functional activity. In particular a study of the variations in the appearances of the specific cell types during the breeding cycle would be valuable.

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THE ACTION OF FLUOTHANE, CHLOROFORM AND HYPOTHERMIA ON THE HEART

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SUMMARY.

Physiological and metabolic changes produced in the isolated heart by fluothane, chloroform and mild hypothermia have been recorded and compared.

Both fluothane and chloroform depressed cardiac work output and efficiency. Hypothermia raised the level of efficiency.

Chloroform, fluothane and hypothermia all decreased the myocardial rate of oxygen consumption.

Fluothane apparently decreased the sensitivity of the isolated heart to both adrenaline and nor-adrenaline.

INTRODUCTION.

Raventos (1956) demonstrated the anaesthetic properties of the non-inflammable, volatile liquid fluothane ($CF_3CHClBr$) and showed that whilst anaesthesia induced by the inhalation of this fluorohydrocarbon was comparatively free from cardiac irregularities it was consistently accompanied by hypotension. He noted that the fall in blood pressure was roughly proportional to the concentrations of the vapour used and found a marked species variation in the degree of hypotension produced.

Severenghaus and Cullen (1958) confirmed and extended Raventos' observations, reporting a reduction in cardiac output and arterial pressure during fluothane anaesthesia which they believed to be due primarily to myocardial depression. Further, they recorded a fall in total body oxygen consumption of 15-20 p.c. after breathing 1-5 p.c. fluothane in air, indicating the possibility that the physiological depression was accompanied by metabolic changes. Stephen *et al.* (1958) confirmed these latter results, recording a depression of respiratory tidal volume during fluothane anaesthesia.

Because of its hypotensive effect fluothane has recently been used as an anaesthetic during cardiac surgery where a mild degree of myocardial depres-

[1] The expenses of this investigation were defrayed by a grant from the Life Insurance Medical Research Fund of Australia and New Zealand.

sion and lowered cardiac output is desirable provided that it is not associated with a marked fall in cardiac efficiency.

The present study was made in an attempt to record some of the physiological changes produced on the isolated spontaneously beating heart by fluothane, and in particular to determine whether or not any of the fluothane-induced hypotension is of direct cardiac origin and, if so, whether it is associated with a significant alteration in cardiac efficiency. The claim that heparin potentiates the fluothane-induced myocardial depression (Orton, unpublished results) was also investigated.

Since anaesthetic agents other than fluothane induce hypotension and myocardial depression (Malt, 1958; Price *et al.*, 1955; Brewster *et al.*, 1953) the effect of fluothane on the isolated heart has been compared with that of chloroform at a similar dose level. Finally, the cardiac effects of both fluothane and chloroform were compared with those produced by mild hypothermia as used by some workers during cardiac surgery.

Methods.

The isolated spontaneously beating heart of the toad (*Bufo marinus*) was used throughout this study as previously described (Nayler and McKelvie, 1956). Thus a recycling heart perfused with toad Ringer solution was placed in a modified Warburg apparatus and beat rate, number of drops ejected per beat, maximum aortic pressure, rate of oxygen utilization and carbon dioxide production and E.C.G. tracings recorded simultaneously and repeatedly throughout the three-hour perfusion period. Following temperature equilibration a control perfusion was always recorded for one hour before the anaesthetic agent being studied was added to the perfusate. The vessel was oxygenated at the beginning of the experiment and, unless otherwise stated, all observations were made at $25{\cdot}0 \pm 0{\cdot}05°$ C. Unselected winter male and female toads were used.

From the data obtained the total work output of the heart in gram-cm./minute was calculated and, by reference to the rate of oxygen utilization, an estimate of overall efficiency derived.

Fluothane.[2] In a series of six experiments fluothane was added to the perfusate (final concentration 1 in 10^{-3}) after the preliminary hour's perfusion and observations continued throughout the ensuing two hours. In a further series of six experiments heparin[3] was initially added to the perfusate (final concentration 0·1 units/5 ml.) and fluothane added as above. In both series observations were made at fifteen-minute intervals throughout the perfusion except in that period immediately following the addition of the drug, when three consecutive five-minute readings were taken.

Chloroform.[4] In a similar series chloroform was added to the perfusate after the initial one hour's perfusion to give final concentrations of 1 in 10^{-3} and 1 in 10^{-2}, six experiments being performed at each concentration. Observations were made as above both before and after the addition of the drug to the perfusion system.

Thus Ringer solutions saturated with either fluothane or chloroform were prepared by mechanical shaking for thirty minutes and the appropriate dilutions made in Ringer from this saturated stock solution (Raventos, 1956).

Hypothermia. Since hypothermia has a recognized role in cardiac surgery some ob-

[2] Fluothane: I.C.I. product. [3] Heparin: Boots Pure Drug Co. [4] Chloroform: Anaesthetic.

servations on the effect of a 5° C. fall in temperature on the isolated heart were recorded.

A series of six hearts were initially perfused for one hour at 25·0° C. and the temperature then rapidly reduced to 20·0° C. Changes in beat rate, number of drops ejected per beat, maximum aortic pressure, carbon dioxide output and oxygen uptake rates were recorded as above and from these data the relative efficiency of the isolated heart at 25·0° C. and 20·0° C. calculated.

Control series. A series of six control experiments were performed at both 25·0° C. and 20·0° C. in which observations were made at fifteen-minute intervals throughout the three hours' perfusion.

Response to pressor amines. As an appendage to this study some kymograph records of the action of adrenaline[5] and nor-adrenaline[6] on fluothane treated hearts were made in an attempt to determine whether a reduced sensitivity to the amines was responsible, in part at least, for the claimed reduction in cardiac irritability under fluothane anaesthesia. Following the addition of fluothane (final concentration 1 in 10^{-3}) nor-adrenaline (0·01 μgm.) was added to a series of recycling hearts and the change in force of ventricular contraction recorded on the kymograph compared with that recorded following the addition of nor-adrenaline at the same dose level to fluothane-free preparations. In a similar series adrenaline (0·02 μgm.) was added to fluothane treated hearts. Dilutions of adrenaline and nor-adrenaline were prepared in Ringer in the presence of ascorbic acid.

RESULTS.

The various parameters of cardiac physiology and metabolism recorded in this study showed only minor variations in both control series, a slow, steady decline in maximum aortic pressure and cardiac efficiency being recorded at both temperatures. By contrast both fluothane and chloroform produced marked myocardial depression resulting in reduced levels of useful work output and lowered cardiac efficiency. The 5° C. drop in perfusion temperature resulted in an actual increase in cardiac efficiency. These results are summarized in Figs. 1-7, where each graph represents the mean of six experiments.

Maximum pressure. Fluothane (1 in 10^{-3}) produced an immediate and marked fall in maximum aortic pressure which was not modified by the addition of heparin. Thus in the results summarized in Fig. 1a fluothane has reduced the maximum aortic pressure by 45 p.c. in both heparin free and heparin treated preparations.

Similarly, chloroform (1 in 10^{-3}) produced an immediate reduction of 20 p.c. in the aortic pressure and at higher concentration the pressure was further reduced—see Fig. 1b. In both the fluothane and chloroform series the reduced pressure level was maintained for approximately thirty minutes after which time there was a gradual return to normal levels.

Reduction of the perfusion temperature from 25 to 20·0° C. resulted in a slight increase in aortic pressure—Fig. 1b. This raised pressure is probably associated with the pronounced bradycardia induced by the lowered temperature —Fig. 2b.

Beat rate. At the concentration used throughout this series fluothane failed

[5] Adrenaline: as base Adrenaline B.P. [6] Nor-adrenaline: as Noradrenaline Bitartrate.

to produce any significant alteration in beat rate—Fig. 2a. Higher concentrations, however (1 in 10^{-2}) consistently induced marked bradycardia, as can be seen in the lower E.C.G. tracing shown in Fig. 11. Both reduction of the perfusion temperature and the administration of chloroform (1 in 10^{-3}) produced well sustained bradycardia—Fig. 2b.

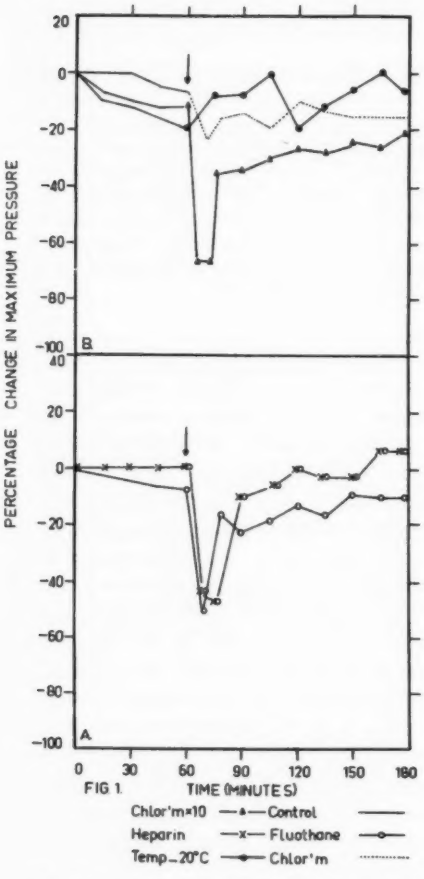

FIG 1.

Fig. 1. (a) Effect of fluothane (1 in 10^{-3} final concentration) on aortic pressures recorded from untreated and heparinised (0·1 units/5 ml.) hearts. (b) Effect of chloroform (1 in 10^{-3} and 1 in 10^{-2} final concentration) and of 5° C. reduction in perfusion temperature on aortic pressures. In all cases the mean results of six experiments are displayed and expressed as the percentage change relative to the initial pressures recorded.

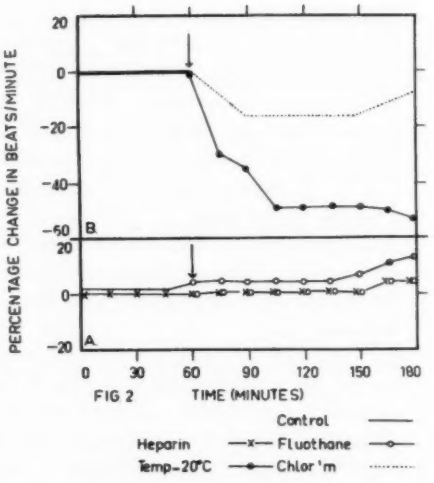

FIG 2

Fig. 2. (a) Effect of fluothane (1 in 10^{-2} final concentration) on the beat rate of untreated and heparinised hearts. (b) Effect of chloroform (1 in 10^{-3} final concentration) and 5° C. fall in temperature on beat rate. The results, expressed as the percentage change, are the mean results of six experiments.

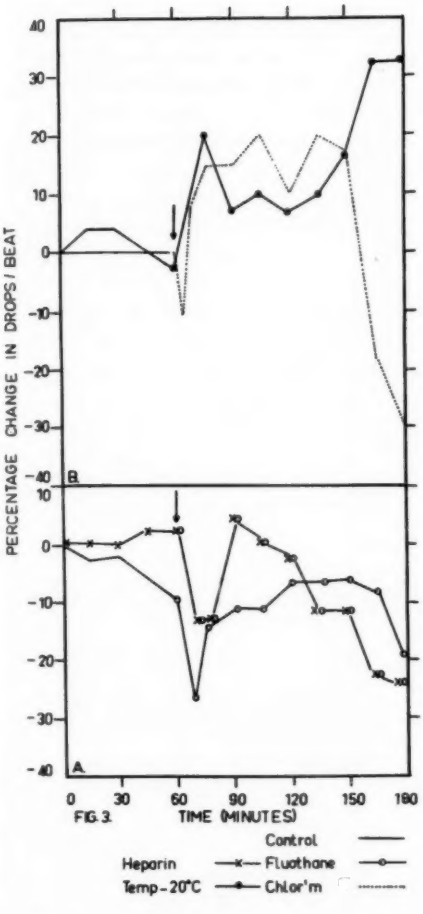

Fig. 3. (a) Effect of fluothane (1 in 10^{-3} final concentration) on the number of drops per beat ejected by heparinised and untreated hearts. (b) Effect on the number of drops per beat of a decrease in temperature (5° C.) and of chloroform (1 in 10^{-3} final concentration). Each graph represents the mean results of six experiments.

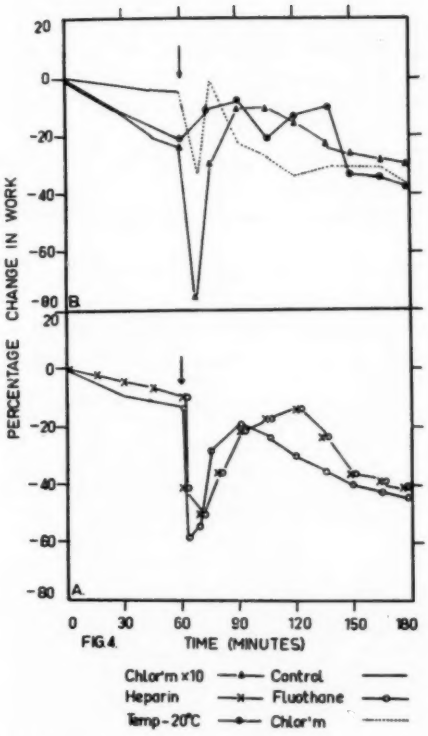

Fig. 4. (a) Effect of fluothane (1 in 10^{-3} final concentration) on the work output of untreated and heparinised hearts. (b) Effect of a 5° C. reduction in temperature and of chloroform (1 in 10^{-3} and 1 in 10^{-2} final concentration) on the cardiac work output. Each graph represents the mean results of six experiments, expressed as the percentage change.

Drops per beat. Fluothane (1 in 10^{-3}) both in the presence and absence of heparin resulted in a fall of approximately 15 p.c. in stroke volume as represented by the number of drops per beat—Fig. 3a. Chloroform (1 in 10^{-3}) produced only a transient fall followed by an increased output per beat, probably

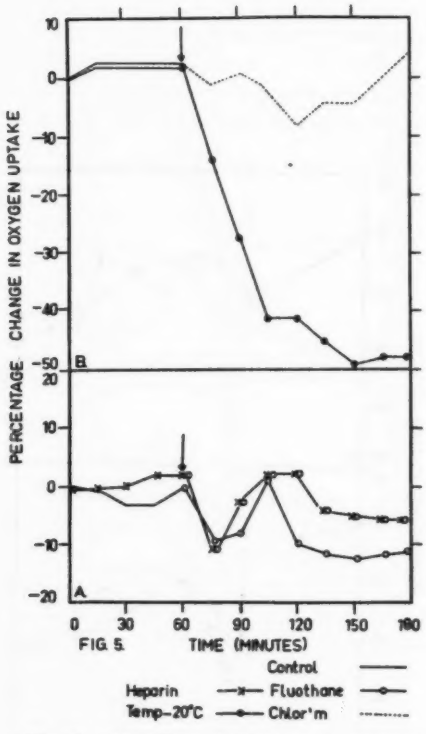

FIG. 5.

Fig. 5. (a) Effect of fluothane (1 in 10^{-3} final concentration) on rate of oxygen uptake by untreated and heparinised hearts. (b) Effect of 5° C. fall in temperature and of chloroform (1 in 10^{-3} final concentration) on the rate of oxygen uptake. The mean results of six experiments are displayed in each graph.

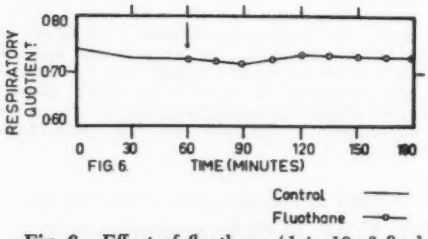

FIG. 6.

Fig. 6. Effect of fluothane (1 in 10^{-3} final concentration) on the respiratory quotients of winter isolated hearts. Mean results of six experiments are shown.

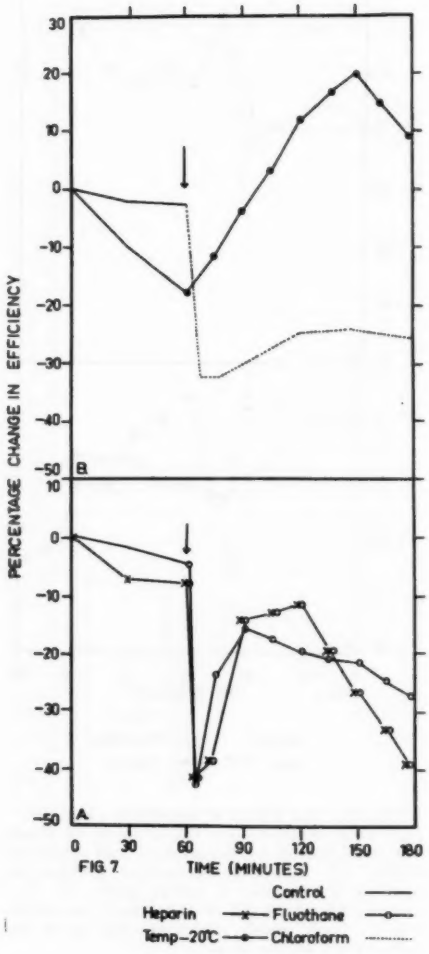

FIG. 7.

Fig. 7. (a) Effect of fluothane (1 in 10^{-3} final concentration) on the efficiency of untreated and heparinised hearts. (b) Effect of chloroform (1 in 10^{-3} final concentration) and of mild hypothermia on the efficiency of the isolated heart. In each case the mean results of six experiments are displayed.

associated with the attendant bradycardia noted in Fig. 2b. As with chloroform, hypothermia resulted in an increased number of drops per beat, again associated with bradycardia.

Work output. Correlating the data displayed in Figs. 1-3, an index of the useful work done by the heart before and after the addition of the anaesthetic agents or the reduction in perfusion temperature was calculated.

As can be seen in Fig. 4a and b, both fluothane and chloroform reduced the work output of the heart used in this preparation, and heparin made no appreciable difference to the fluothane effect. In contrast, hypothermia produced a small increase in the work done by the heart, despite the pronounced bradycardia already commented upon.

Oxygen uptake and respiratory quotient. In Fig. 5a and b the effect of fluothane (1 in 10^{-3}) in the presence and absence of heparin, of chloroform (1 in 10^{-3}) and of hypothermia on the rates of oxygen uptake by the isolated heart are summarized.

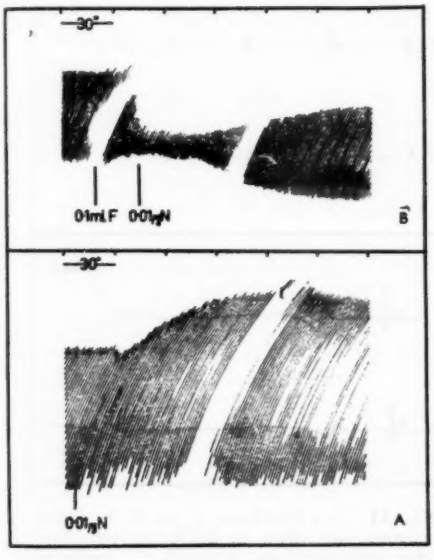

Fig. 8. Kymograph tracings showing the effect of 0·01 μg. nor-adrenaline on recycling hearts perfused in presence (b) and absence (a) of fluothane (final concentration 1 in 10^{-3}).

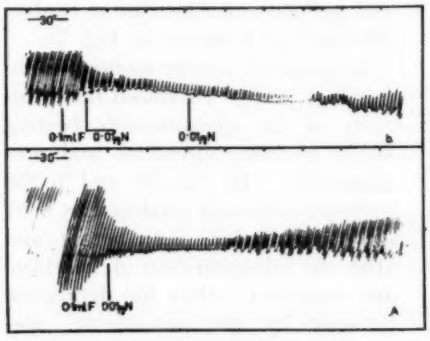

Fig. 9. (a) and (b) Kymograph records showing effect of nor-adrenaline on two hearts perfused in presence of fluothane (1 in 10^{-3} final concentration). Note absence of true inotropic response.

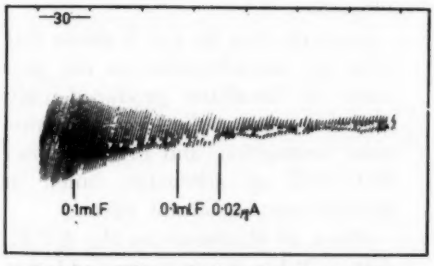

Fig. 10. Effect of adrenaline (0·02 μg.) on the isolated heart perfused in presence of fluothane (1 in 10^{-3} final concentration)—kymograph record.

Both anaesthetic agents depressed the rate of oxygen uptake by the isolated heart, but the most pronounced fall was that recorded following the reduction in perfusion temperature. The 5° C. drop in temperature resulted in a 50 p.c. fall in the rate of oxygen utilization.

Although fluothane depressed the rate of oxygen uptake it failed significantly to alter the respiratory quotient of the isolated winter heart—see Fig. 6.

Efficiency. Correlating the work output data with the altered rates of oxygen consumption provided indices of cardiac efficiency which are displayed in Fig. 7a and b. In both the fluothane series this anaesthetic at the concentration used reduced the cardiac efficiency by approximately 40 p.c. After an initial sharp fall in efficiency following the addition of the drug to the perfusate there was a gradual return to normal. Similarly, the administration of chloroform (1 in 10^{-3}) resulted in a sharp decline in efficiency which appeared to be maintained over a longer time interval than was observed with fluothane.

Hypothermia resulted in a marked and well-sustained increase in cardiac efficiency, as is shown in Fig. 7b.

Response to pressor amines. Fluothane (1 in 10^{-3}) reduced the sensitivity of the spontaneously beating hearts to both adrenaline and noradrenaline. In Fig. 8a and b the inotropic responses produced by 0·01 μg. nor-adrenaline both before and after the administration of fluothane are compared. After the depression induced by the anaesthetic, noradrenaline produced only a small increase in contractile force (Fig. 8b) when compared with the response recorded from the untreated heart (Fig. 8a).

Similarly, Fig. 9a and b shows that 0·01 μg. nor-adrenaline in the presence of fluothane produced only minor changes in the force of ventricular contraction; and Fig. 10 shows that 0·02 μg. adrenaline failed to produce any significant effect.

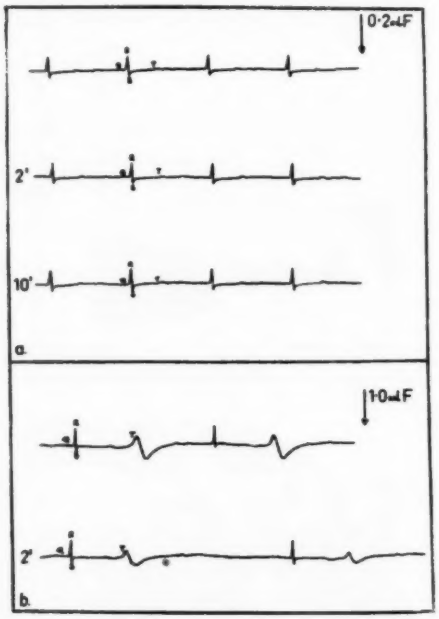

Fig. 11. (a) Fluothane (1 in 10^{-3}) failed to produce any effect on E.C.G. record. (b) Higher concentrations (1 in 10^{-2}) produced bradycardia and depression of the 'T' wave.

Effect of fluothane on the E.C.G. pattern. When fluothane was added at concentrations sufficient to produce myocardial depression without bradycardia, no change was noted in the E.C.G. pattern recorded from the isolated heart

(see Fig. 11a) where fluothane was added to give final concentration 1 in 10^{-3}. At concentrations sufficiently high to produce bradycardia (1 in 10^{-2}) a reduction in the 'T' wave was consistently recorded—Fig. 11b.

DISCUSSION.

The data obtained from this study point to a similar mode of action for both fluothane and chloroform. Both depressed myocardial contractility, reduced the rate of oxygen utilization, lowered the useful work output and resulted in a reduction in efficiency of the isolated cardiac preparation. Heparin failed to exert any modifying influence on either the physiological or metabolic changes produced by the administration of fluothane.

In contrast, mild hypothermia resulted in a significant and well-sustained raised level of efficiency which was associated with a depressed rate of oxygen uptake.

In general, these results are in agreement with those of other workers who have investigated some of the physiological changes associated with fluothane anaesthesia in intact animals. Severenghaus and Cullen (1958) noted a reduction in cardiac output, arterial pressure and stroke volume during fluothane anaesthesia and concluded that these effects were mainly due to myocardial depression. These workers also reported a reduction of 15 p.c. in total oxygen consumption, thereby indicating that some metabolic changes were associated with the observed physiological effects. Since no change in respiratory quotient accompanied the depressed level of oxygen consumption recorded in the above study, it seems probable that a depression rather than an actual change in cardiac metabolism has taken place.

Similarly, Stephen *et al.* (1958) noted hypotension during the induction of fluothane anaesthesia, and suggested that peripheral vasodilatation as well as reduced myocardial output may have been involved. Although the above data provide no information on possible peripheral activity there is little doubt that direct cardiac depression is involved in the reported hypotensive effects of fluothane.

Raventos (1956) reported that bradycardia was associated with the hypotensive effects of fluothane in both cats and dogs. Since in the present series bradycardia was only found to be associated with high doses of fluothane the reduced beat rate recorded by Raventos may have been of reflex origin. It should be noted that in the preparation used only a small dose range existed between that concentration of fluothane which produced slight hypotension and that which resulted in gross bradycardia or cardiac arrest.

Other anaesthetic agents besides fluothane are known to depress myocardial contractility. Thus Price and Helrich (1955) reported that cyclopropane, diethyl ether and thiopental all have depressant effects on the heart which are proportional to their concentration. In 1953, Brewster *et al.* noted that the

depressant effect of ether on the dog myocardium was modified by the reflex release of adrenaline and nor-adrenaline. Since the isolated heart showed reduced sensitivity to both amines during fluothane anaesthesia it seems unlikely that the above results are confused by the reflex liberation of nor-adrenaline from the myocardium. Raventos recorded cardiac irregularities following the administration of adrenaline to dogs during fluothane anaesthesia, and in some cases he recorded fatal ventricular fibrillation. The discrepancy between the results reported and those of Raventos is possibly due to differences in the concentrations of fluothane since deep chloroform anaesthesia is known to reduce the sensitivity of the heart to both adrenaline and nor-adrenaline, whilst cardiac irregularities have been repeatedly reported during light chloroform anaesthesia.

Progressive bradycardia, reduced oxygen consumption and increased mechanical efficiency during hypothermia have been reported by earlier workers. Badeer (1956, 1958) using a denervated dog's heart doing constant work in a modified heart-lung preparation found that a reduction in temperature from 36° C. to 26° C. was associated with increased efficiency. He concluded that cold did not interfere with the conversion of aerobic energy into useful work. Although a reduction in cardiac output was not recorded in the present work where a temperature drop of only 5° C. was used, other workers using larger temperature variations have noted reduced cardiac output, together with increased levels of efficiency (Berne, 1954).

Acknowledgment. My thanks are due to Dr. T. E. Lowe for his help with, and interest in, this project.

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THE ADSORPTION OF I^{131} IODIDE IONS ON PLASMA PROTEINS

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(*Accepted for publication 13th February, 1959.*)

SUMMARY.

Results are reported which are not consistent with an assumption of covalent binding between I^{131} ions and plasma protein molecules, when KI^{131} is incubated *in vitro* with plasma.

Within the limits of accuracy of the observations (about ± 1 p.c.), there is no evidence for the adsorption of I^{131} ions to plasma proteins at concentrations of I^{131} normally used in human tracer studies.

Any error in the estimate of extracellular fluid volume using I^{131}, arising from the attachment of I^{131} ions to plasma protein, is therefore not greater than about 1 p.c.

Approximately 12 p.c. of the radioactivity of the radio-iodinated human serum albumin, as received, was due to freely dialysable iodide ions, and this fraction does not vary appreciably with time.

INTRODUCTION.

Recently a technique for measuring the extracellular fluid volume in human subjects has been developed at this hospital (George, Seldon and Hickie, 1959). In this method, a measured amount of I^{131} is injected intravenously in subjects whose thyroidal iodine uptake has been temporarily blocked by the oral administration of large amounts of potassium iodide. The injected I^{131} rapidly diffuses through the capillary walls into the extracellular fluid, reaching an equilibrium concentration in about one hour. From the plasma concentration, that in the extracellular fluid is calculated by taking into consideration the two following effects: (i) the reduced space occupied by the water in the plasma, and (ii) the Donnan shift occasioned by the higher protein concentration in the plasma compared with that in the extracellular fluid. From the corrected concentrations the extracellular fluid volume is determined.

A third mechanism that might cause a possible difference in the relative equilibrium concentrations should be considered, namely, the attachment of I^{131} ions to plasma protein molecules, either by chemical binding or by adsorption. An investigation has been carried out in order to determine the magnitude of this effect in order to be able to apply such corrections in the extracellular volume work as may be necessary. The results of this investigation are reported

in this paper. In the course of the investigation the same techniques were used to separate the free and bound iodide in radio-iodinated human serum albumin and the results are mentioned briefly.

RESULTS.

The following investigations were carried out on samples of sterile human plasma to which KI^{131} had been added in a concentration similar to that involved in the measurement of extracellular volume, namely, 1 to 10 μc/litre:

(a) Precipitation of the serum protein with trichloracetic acid.

(b) Compensation dialysis.

(c) Ion extraction using De-acidite FF ion exchange resin (bromide form).

The precipitation with trichloracetic acid denatures the protein and consequently the magnitude of the protein effects cannot be estimated by this method. This objection does not apply in the case of covalent bonding, as this is independent of the protein surface. It was for this reason that the investigations (b) and (c) were carried out.

Precipitation with trichloracetic acid.

0·1 μc of I^{131} was added to 10 ml. of plasma. After standing for six hours, 10 ml. of trichloracetic acid were added. The radioactivity of a 10 ml. aliquot of the supernatant was measured with a scintillation counter. The results are

TABLE 1.

c.p.m./ml. plasma	1396
c.p.m./ml. supernatant	1442
c.p.m./ml. supernatant, corrected for plasma water	1399

given in Table 1, and when corrected for the protein volume, show that no chemical combination between the I^{131} and the protein molecules had taken place.

Compensation dialysis.

Plasma was dialysed against an isotonic solution of NaCl, each solution containing an equal tracer concentration of I^{131}. It was thought that if adsorption on the protein occurred, an increased radioactivity in the plasma phase would be observed.

After a 19-hour dialysis, the concentration of the I^{131} was found to be 7 p.c. higher than that in the dialysate, individual values of two samples being 6·5 p.c. and 7·8 p.c.

One interpretation of these results would be that the iodide ion is adsorbed in preference to the Cl^- and HCO_3^- ions, these being the anions in human plasma.

An alternative explanation for this shift may be considered in terms of a difference in the ionic activity, A, on each side of the membrane. That is, the effective ionic concentration is smaller in the plasma solution. A is related to the stoichometric concentration by the relationship: $A = \gamma c$, where γ is the activity coefficient.

In order to distinguish between these two interpretations, the dialysis was repeated with a large excess of KI^{127} added in equal concentration to each side of the membrane. This increased the iodide concentration by a factor of about 1000. Again the concentration of the I^{131} was found to be higher in the plasma, this time the increase being 10 p.c., a figure not significantly different within the limits of experimental error from that found without the addition of carrier iodide. Had preferential adsorption of the iodine ion been the cause of the observed shift, then the adsorption of the I^{131} ions should have been negligible in the second case, as a result of the swamping action of the carrier iodide. As this is not the case, the alternative explanation in terms of a reduced activity of the iodide ions seems to be a likely explanation of the observations.

Resin extraction.

The lack of adsorption of I^{131} ions on plasma proteins was checked in another way, using ion-exchange resin to extract the iodide ions. It was argued that if ions were attached by ionic bonds to the protein, they would be extracted from solution by the ion-exchange resin more slowly than if they were all free ions. Thus, while the final degree of extraction by the resin may be the same in both cases, the rate of extraction expressed as percentage extracted per minute would be lower if adsorption took place.

For this purpose, 10 ml. of solution were agitated with 2 gm. of De-acidite FF ion exchange resin, bromide form, for one minute. 5 ml. of supernatant were assayed with a scintillation counter and the percentage extraction per minute calculated.

The following solutions were measured 6 hrs. after the addition of KI^{131}: (i) Human plasma to which KI^{131} had been added at a concentration of 10 μcs litre. (ii) A solution of 103m Eqs/litre NaCl plus 30m Eqs/litre $NaHCO_3$

TABLE 2.

Rate of extraction of iodide ions by exchange resin. The results are expressed as percentage extraction per minute.

Sample	Plasma + KI^{131}	NaCl + $NaHCO_3$ + KI^{131}	Distilled water + KI^{131}
1	90·8	90	98·6
2	90·0	90·5	98·9
3	90·5	—	98·7
Mean	90·4	90·2	98·7
S.D.	± 0·4	± 0·4	± 0·2

and these being the same concentrations as exist in human plasma, plus KI^{131} in the same concentration as in (i). (iii) Distilled water plus the same concentration of KI^{131} as (i) and (ii). The results are shown in Table 2.

From Table 2 it is seen that the rate of extraction in plasma, 90·4 p.c. per minute, is the same as that in a solution of NaCl and $NaHCO_3$ (90·2 p.c.) within the limits of experimental error. Thus there is no evidence for any significant degree of adsorption of I^{131} by the plasma proteins. It is of interest to note that these rates of extraction are both lower than that obtained with distilled water, where the rate was 98·7 p.c. per minute. This is consistent with the expected behaviour of the exchange resin.

In all of the above solutions the extraction measurements were repeated on the supernatants, and in every case the extraction rates obtained agreed with those given in Table 2. This process was repeated several times with the same results, showing that, for the solutions in Table 2, the radioactivity decreased exponentially with time following the addition of the resin, indicating that a single type of process was involved.

Separation of bound and free iodide in iodinated serum albumin.

In the course of this work, some extraction experiments were carried out with radio-iodinated human serum albumin (RIHSA) supplied by the Commonwealth X-Ray and Radium Laboratories and obtained from the Radiochemical Centre, Amersham. In this case the I^{131} is bound by a covalent bond in the benzene ring of the tyrosine in the protein (McFarlane, 1958), and hence the extraction of I^{131} by the resin should be negligible.

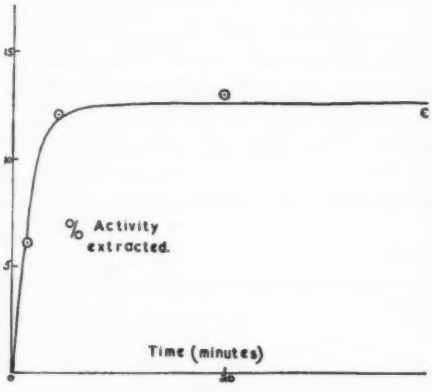

Fig. 1.

In carrying out the extractions, the RIHSA was diluted in carrier albumin in order to minimise surface effects due to the walls of the container or the resin. 0·1 μc RIHSA was added to 5 ml. plasma and made up to 10 ml. with distilled water, for ease of handling. In this case the total free I^{131} ions found in three separate determinations were 11, 10 and 12·5 p.c., giving a mean of 11·2 p.c. ± 1·2 p.c.

Continued extraction of one of these samples followed the curve shown in Fig. 1, and indicated that saturation was reached in the amount of removable I^{131} at about 12·5 p.c. ± 2 p.c.

These results were confirmed by dialysing samples of RIHSA in a solution

of NaCl and $NaHCO_3$ at a concentration equal to that in plasma, namely, 6·0 gm./litre NaCl, 2·27 gm./litre $NaHCO_3$. It was found that four separate batches of RIHSA of various ages, contained 10, 11, 10 and 12 p.c. free I^{131}, a mean of $10·8 \pm 1·0$ p.c. These results were consistent with those found by the resin method, thereby supporting the validity of the resin extraction procedure.

The conclusions reached from this dialysis experiment were substantiated by precipitating the RIHSA with trichloracetic acid. Identical results were obtained with both methods.

DISCUSSION.

From the first experiment it was concluded that the iodide ion does not enter into chemical combination with protein molecules when added to plasma. From the remaining experiments it was concluded that any adsorption which takes place is smaller than the limits of experimental error which are about ± 1 p.c. in this case.

There is no doubt that some small degree of ionic attachment on protein does occur due to the action of Coulomb and Van der Waal type forces. In either case a dynamic adsorption equilibrium would be established. Under these circumstances the adsorption process is competitive and the different ions in plasma would be adsorbed approximately proportionally to their plasma concentrations. As the adsorption surface of the protein is limited by the number of free positive centres on it, one would not expect significant adsorption of the I^{131} ions, which are present in our solutions in very low concentrations relative to the other anions present, namely, 10^{-12} gm./litre of I^{131} compared with several gm./litre of chloride and bicarbonate.

Another factor tending to reduce the adsorption of I^{131} in plasma proteins is the fact that the pH of plasma is about 7·5, a value at which the protein has a net negative charge. The negative state of the protein would be expected to reduce adsorption of anions to a very low level and our observations are consistent with this view.

On the conclusion of this investigation our attention was drawn to the work of Lybeck (1957) who added I^{131} to serum and then separated the mixture electrophoretically. On completion of the electrophoresis, the paper strips were dried and scanned for radioactivity. In no case could any radioactivity be detected in the protein area.

Acknowledgments. The RIHSA and KI^{131} were supplied by the Commonwealth X-Ray and Radium Laboratory, whom we would like to thank. Drs. D. Rothfield and J. Garvan suggested to us the use of the ion exchange resin, and we wish to thank them for many discussions. We would like to express our appreciation of the helpful suggestions received from Dr. A. S. McFarlane and Dr. Dora Winikoff. We are grateful to the Sisters of Charity for the provision of facilities for doing this work, and to Professor Messel and the Nuclear Research Foundation of the University of Sydney for the loan of some of the equipment. The balance of the equipment was provided by the State Cancer Council.

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THE EFFECT OF THE ANTIOXIDANT BUTYLATED HYDROXY TOLUENE ON SERUM LIPID AND GLYCOPROTEIN LEVELS IN THE RAT

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SUMMARY.

Observations were made of the effects of variations in the dietary content of lard and butylated hydroxy toluene (BHT) on total serum cholesterol, phospholipid, total esterified fatty acid, mucoprotein and hexosamine levels of male rats in a paired feeding experiment, and of female rats in a chronic study.

A concentration of 0·001 p.c. BHT (dry weight) caused no changes in any of the serum constituents studied.

0·1 p.c. BHT, with 10 p.c. lard supplement to the diet, increased the serum cholesterol levels of female rats in the chronic study, but no other significant changes were produced.

0·5 p.c. BHT produced increases in the serum cholesterol levels of male rats within five weeks, and serum cholesterol, phospholipid and mucoprotein levels in female rats after eight months.

A 10 p.c. and 20 p.c. lard supplement to the basic diet produced significant reductions in the serum cholesterol, phospholipid and hexosamine levels of *ad lib.* fed male rats within five weeks.

Restriction of food intake during the first two weeks, on a basic diet unsupplemented with lard, produced significant reductions in serum cholesterol and phospholipid levels in male rats after five weeks.

The serum levels of total esterified fatty acids were not affected by any of the treatments applied.

A possible relationship between the metabolism of relatively large doses of BHT, the fatty acids and the transport of cholesterol and phospholipid is suggested.

INTRODUCTION.

Butylated hydroxy toluene (2:6-di-*t*-butyl-p-cresol, referred to as BHT) was introduced as an antioxidant to delay the onset of rancidity in foodstuffs for human consumption (Dugan *et al.*, 1954). Although this antioxidant has been

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in use for some time, very little information is available concerning its metabolism *in vivo*. It has been shown that a proportion of an ingested dose of BHT is excreted in the urine as glucuronides and ethereal sulphates (Dacre, 1957; Deichmann *et al.*, 1955). Johnson *et al.* (1958a) have shown that in the rat a small percentage of ingested BHT is probably deposited in the perirenal fat increasing its chemical stability, and reducing its iodine value.

The present paper describes the effect of BHT on serum lipid and glycoprotein levels, and suggests that relatively large doses of BHT interfere with the metabolism of those fatty acids involved in the transport of both total serum cholesterol and phospholipid.

Materials and Methods.

Paired feeding experiment.

Forty-five pairs of male weanling litter mates, of the Norway Hooded strain of rat, were selected from litters of our stock colony, on a basis of the closest possible approximation of their weights.

The 45 pairs were randomly subdivided into five groups, avoiding the use of two litter pairs from the same original litter within the same group. The five groups represented five replicates within the experimental design. These replicates were treated as a unit, i.e. they were sacrificed and sera samples taken and analysed under the same conditions, to ensure valid comparisons.

The animals were housed singly, one of the sibs of each pair being pair-fed against its litter mate. The nine litter pairs within one group were randomly allocated to the nine pairs of diets within one replicate (Table 1).

TABLE 1.

Lard and BHT content of diets.

Percentage lard supplement to stock diet (dry weight)	Percentage BHT		Percentage BHT		Percentage BHT	
	Ad lib. feeding	Pair-fed	*Ad lib.* feeding	Pair-fed	*Ad lib.* feeding	Pair-fed
0	0	0·001	0·1	0	0·5	0
10	0[a]	0·001	0·1[a]	0	0·5[a]	0
20	0[b]	0·001	0·1[b]	0	0·5[b]	0

(a) and (b) Diets also used in chronic study.

A further nine weanling male litter pairs, of the Albino Wistar strain of rat, all of approximately the same weight, were randomly allocated to the nine groups within the experiment. The results from these 18 animals did not differ significantly from those of the Norway Hooded strain and the results of both strains of rat were pooled for statistical analyses.

The conventional methods of the paired feeding technique were employed, the animals within the first cage of each diet pair (Table 1) being fed *ad lib.* and their food consumption measured to 0·1 gm. On the succeeding day its litter mate was fed the same amount of the appropriate diet.

The stock diet and the method of preparation of the experimental diets containing 10 and 20 p.c. lard supplement have been described previously (Johnson *et al.*, 1958a). The BHT[3] was added to the diet unsupplemented with lard by dry homogenisation in a Waring blender in the appropriate proportions of BHT, full cream powdered milk, sodium chloride, and calcium carbonate. The blended material was then mixed with the remainder of the dry materials of the diet, and treated in a similar fashion to the lard-containing diets.

The concentrations of BHT in the lard were so chosen that direct comparisons could be made between the effect of varying amounts of BHT, at different levels of lard supplement to the stock diet, and conversely the effect of a varying lard content of the diet at the same level of BHT.

Five weeks after they were placed on their respective diets, approximately 5 ml. of blood were taken by intracardiac puncture under ether anaesthesia, from the first 18 animals representing nine litter pairs. The animals were then sacrificed by further ether anaesthesia. The clotted blood was centrifuged at 3,000 r.p.m. for 10 minutes. The sera were analysed in duplicate for total cholesterol (Zlatkis *et al.*, 1953), phospholipid (Brown, 1954), and total esterified fatty acids (Stern and Shapiro, 1953). In these determinations 3:1 ethanol ether extracts of the sera were used.

Mucoprotein was determined by the method described by Winzler *et al.* (1948), and hexosamine by the method of West and Clarke (1938). Both methods have been modified as previously described by Schwartz and Gilmore (1958).

Subsequently the other groups of 18 animals were sacrificed at approximately three-day intervals. Thus the time that the animals were on a given diet ranged from five to eight weeks. No differences were found between the sera constituents in the first and last groups.

Chronic study.

A second experiment to evaluate the effects of continuous ingestion of BHT in female rats over an eight-month period, was designed as follows. One batch of three weanling female litter mates, of the Norway Hooded strain of rat, were randomly distributed between the 10 p.c. lard supplemented diets containing 0, 0·1 and 0·5 p.c. BHT (see a, Table 1), and a second between the 20 p.c. lard supplemented diets containing the same amount of BHT (see b, Table 1). This procedure was repeated eight times, so that on each diet there were eight animals caged in two groups of four. In all experiments to be described the various litters were treated as a unit to ensure valid comparisons.

The diets were supplied *ad lib.* during the whole course of the experiment. After five months of treatment the animals were mated with similarly fed litter mates, as part of a reproduction study (Johnson and O'Halloran, 1958b). At least six weeks after weaning of the young, the female rats were sacrificed and the sera examined as outlined above.

RESULTS.

Paired feeding experiment—Control animals. On each lard level there were three groups of control animals; those being fed *ad lib.*, those being fed the same amount of food as their litter mates whose diet contained 0·1 p.c. BHT, and similarly those pair-fed against animals whose diet contained 0·5 p.c. BHT. Thus, the only differences between the groups of control animals on the same lard supplement were in the amounts of food consumed, and any other effects arising from a limited food supply. These differences were determined by the effect of BHT on the food consumption of their litter mates. The pair-fed controls consumed virtually the same amounts of food as did those animals whose diet contained BHT.

[3] Tenox BHT.

During the first week, the introduction of 0·1 p.c. and 0·5 p.c. BHT to the diet significantly reduced ($p < 0·001$) the food consumption irrespective of the level of lard supplementation. By the fifth week there were no significant differences in the food consumption even when the diet contained 0·5 p.c. BHT (Fig. 1), the rats presumably having become "adapted" to the hot and peppery nature of the BHT-containing diets.

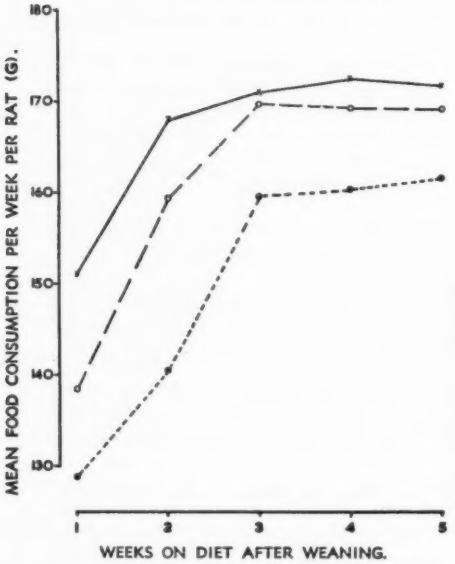

Fig. 1. *The effect of dietary BHT upon the food consumption of male weanling rats.*

×———× 0·0 p.c. BHT.
○----○ 0·1 p.c. BHT.
●. . . .● 0·5 p.c. BHT.

Note: Standard error of a mean (S.E.M.) for week I = 2·1 on 20 degrees of freedom (d.f.). S.E.M. for week 5 = 4·3 on 20 d.f.

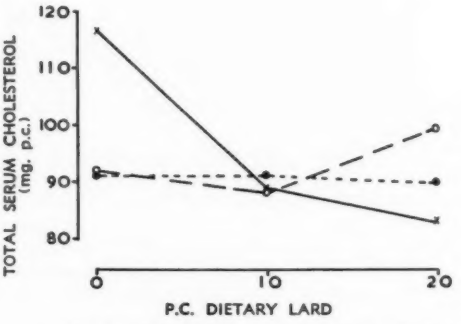

Fig. 2. *The effect of lard and pair-feeding against animals whose diet contained BHT, on the total serum cholesterol levels of male rats.*

×———× *Ad lib.* feeding.
●----● Pair-fed against animals whose diet contained 0·1 p.c. BHT.
○. . . .○ Pair-fed against animals whose diet contained 0·5 p.c. BHT.

S.E.M. = 5·1 on 20 d.f.

The serum mucoprotein and fatty acid levels of the control animals were constant, irrespective of the variation in diet and food intake. However, the total serum cholesterol, phospholipid and hexosamine levels differed significantly between control rats.

Total serum cholesterol (Fig. 2 and Table 2). The supplementation of the stock diet by both 10 p.c. and 20 p.c. lard caused similar reductions in the serum cholesterol levels of *ad lib.* fed male rats after five weeks. No such reductions in the serum cholesterol levels of control rats were observed when the food intake was restricted during the first one or two weeks of the experiment, con-

TABLE 2.

The effect of BHT, lard and pair-feeding on total serum cholesterol levels in the male rat.

			Percentage dietary BHT		
			·001	·1	·5
Percentage dietary lard	0	Treated	102·7[a]	109·5	112·5
		Control	116·5	92·0	91·2
		Difference	− 13·8	17·5	21·3
	10	Treated	97·7	94·0	126·1
		Control	89·0	88·3	90·8
		Difference	8·7	5·7	35·3
	20	Treated	80·7	95·0	142·4
		Control	83·4	100·2	90·2
		Difference	− 2·7	− 5·2	52·2

(a) Mean total serum cholesterol (mg. p.c.) of six animals.

Note: Standard error of mean (S.E.M.) of differences is 10·1 on 20 degrees of freedom (d.f.).

sequent on the paired feeding of the control animals against animals whose diet contained 0·1 p.c. and 0·5 p.c. BHT.

The restriction of the food intake itself had an effect on the serum cholesterol levels of the control animals, and this effect was dependent upon the extent of dietary lard supplementation. Thus a reduction in the level of serum cholesterol was observed when the food was restricted and no lard present in the diet. Under similar conditions of food restriction no alterations were noted when the diet contained 10 p.c. lard, and a raised serum cholesterol was observed with a 20 p.c. lard supplement.

TABLE 3.

The Effect of BHT, lard and pair-feeding on serum phospholipid levels in the male rat.

			Percentage dietary BHT		
			·001	·1	·5
Percentage dietary lard	0	Treated	131·2[a]	136·8	139·2
		Control	141·0	117·8	122·0
		Difference	− 9·8	19·0	17·2
	10	Treated	124·3	115·5	157·7
		Control	116·6	119·5	120·3
		Difference	7·7	− 4·0	37·3
	20	Treated	108·0	128·0	173·3
		Control	109·6	131·0	123·3
		Difference	− 1·6	− 3·0	50·0

(a) Mean serum phospholipid (mg. p.c.) of six animals.

Note: S.E.M. of differences is 12·4 on 20 d.f.

Serum phospholipid (Table 3). The changes of the serum phospholipid of the control rats, upon supplementation of the diet with lard or restriction of the food intake, closely paralleled those of the serum cholesterol.

Serum hexosamine (Table 4). Supplementation of the stock diet by 10 p.c. and 20 p.c. lard produced similar decreases in the serum hexosamine of the control animals.

TABLE 4.

Effect of lard feeding on the serum hexosamine content in male rats.

	Percentage lard supplement to stock diet		
	0	10	20
Hexosamine mg./100ml.	129[a]	121($p<0.01$[b])	122($p<0.01$)

(a) Figures quoted represent means of 18 animals.

(b) p is probability that treatments differ significantly from control.

Note: S.E.M. = 2·2 on 20 d.f.

The effect of BHT on serum constituents.

Paired feeding experiment. In order to eliminate the effects of the significant differences which occurred between groups of control animals, an analysis of variance was made on the difference between each litter pair. BHT at a dietary level of 0·5 p.c. increased the serum cholesterol levels of male rats within five weeks, even when any effects of BHT on the food consumption were eliminated by the use of the paired feeding technique. The increases in serum cholesterol levels were dependent upon the degree of lard supplementation (Table 2 and Fig. 3), an increase in the lard content of the diet enhancing the elevation

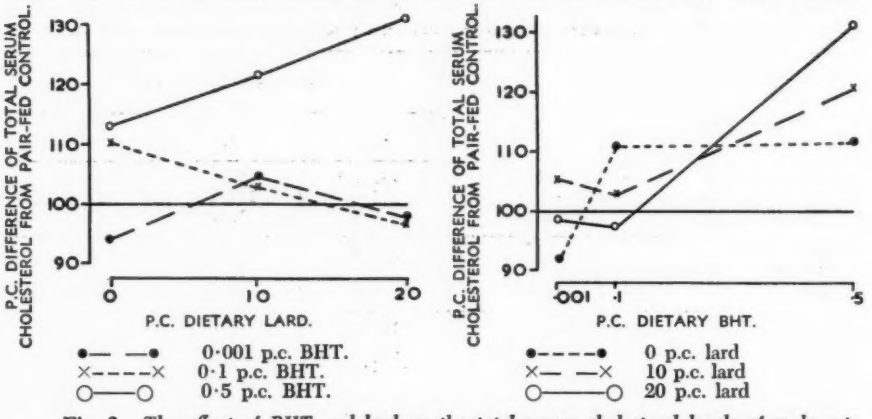

Fig. 3. *The effect of BHT and lard on the total serum cholesterol levels of male rats expressed as a percentage of the serum cholesterol of pair-fed controls.*

of the serum cholesterol levels produced by 0·5 p.c. BHT. Increasing the lard content of a diet containing 0·1 p.c. BHT, antagonises the trend towards an increase in serum cholesterol produced in a non-lard-containing diet. A similar interaction between the lard and the BHT level of the diet on the serum phospholipid levels was noted (Table 3).

TABLE 5.

The effect of BHT on the mean levels of some constituents of the male rat sera.

Determination, mean of 18 animals (mg./100ml.)	Percentage BHT content of diet (dry wght.)						S.E.M. between pair-fed treatment and con-control means on 20 d.f.
	0	0·001	0·1	0	0·5	0	
Fatty Acids	193	190	191	190	208	196	16·1
Mucoprotein	164	155	167	164	170	163	7·5
Hexosamine	125	123	129	124	125	123	4·8

TABLE 6.

The effect of chronic BHT on the mean levels of some constituents of the female rat sera.

Determination (mg./100 ml)	Number of animals	Percentage BHT content of diet (dry weight)			S.E.M. on 4 degrees of freedom
		0	0·1	0·5	
Cholesterol (combined 10 p.c. and 20 p.c. lard supplement)	16	99	100	123($p<0·001$[b])	2·5
Cholesterol (10 p.c. lard supplement)	8	98	109($p<0·05$)	124($p<0·01$)	3·6
Cholesterol (20 p.c. lard supplement)	8	99	92	123($p<0·01$)	3·6
Phospholipid (combined 10 p.c. and 20 p.c. lard supplement)	16	166	163	184($p<0·05$)	4·8
Mucoprotein (combined 10 p.c. and 20 p.c. lard supplement)	16	131	132	160($p<0·01$)	6·7
Fatty Acids (combined 10 p.c. and 20 p.c. lard supplement)	16	190	169	187	8·6
Hexosamine[a] (combined 10 p.c. and 20 p.c. lard supplement)		143	147	161	

(a) Insufficient material for statistical analysis.

(b) Probability of significant difference from appropriate control.

No effects of BHT on the serum content of mucoprotein, hexosamine or total esterified fatty acids were observed (Table 5).

Chronic study.

Further confirmation of the relationship between the level of dietary fat, as a factor in the elevation of serum cholesterol produced by BHT, were observed in the chronic experiment (Table 6). A diet containing 0·1 p.c. BHT and 10 p.c. lard produced significant increases in the serum cholesterol of female rats. At this level of BHT with 20 p.c. lard supplement no increase was observed. A dietary level of 0·5 p.c. BHT produced significant increases in the cholesterol, phospholipid and mucoprotein content of female rat sera. No significant variations in the fatty acid content of the sera were noted on any of the dietary regimens. As insufficient quantities of sera were available for the estimation of the serum hexosamine in each instance, the mean values obtained could not be statistically analysed. However, this component appeared to increase with increased BHT content of the diet, this being consistent with the mucoprotein changes.

DISCUSSION.

The elevations of the serum cholesterol and phospholipid levels observed show that the metabolism of relatively large doses of ingested BHT directly influences the serum levels of these constituents.

That 0·1 p.c. BHT, with a 10 p.c. lard supplement to the diet, produces an increase of serum cholesterol in female rats is of particular interest, as this dose level is referred to as the Normal Testing Dose (NTD) for a food additive. The NTD is that dietary level of a substance which is 100 times the maximum normally expected to be present in foodstuffs for human consumption (Lehman, 1951). One of the major criteria in the determination of the safety of a food additive is that continuous ingestion of the NTD will not cause any departure from normal cellular metabolism.

There is a very close correlation between the levels of cholesterol and phospholipid in serum, and the C/P ratios are constant under a variety of circumstances (Deuel, 1957). Hence the increases in the phospholipid and cholesterol content of the sera in these experiments probably have a common explanation.

It has been shown that the essential fatty acids (EFA) are necessary for the normal transport and metabolism of cholesterol and phospholipid (Kelsey and Longenecker, 1941; Alfin-Slater *et al.*, 1954; Peifer and Holman, 1955). Further clarification of the rôle of fatty acids in the control of the serum cholesterol level has come from the works of Hegsted *et al.* (1957), who have shown that the serum cholesterol level in rats on a cholesterol-cholic acid regimen is inversely related to the products obtained by multiplying the EFA content (linoleic and arachidonic) of the diet by total saturated fatty acid content, the remain-

ing unsaturated fatty acids having a reverse effect in that they increase the cholesterol content of the sera.

It has been established that there is a relationship between the tocopherols, the natural antioxidants and the fatty acids. Thus the abnormal oxidation of highly unsaturated fatty acids within the tissues can be prevented by the *in vivo* antioxidant action of the tocopherols (Dam, 1953), and Kummerow *et al.* (1949) have shown that the tocopherols are functional in the synthesis of oleic and saturated acids.

Whether or not the synthetic antioxidants are metabolically active is debatable. Several synthetic antioxidants can protect an animal from some manifestations of vitamin E deficiency (Dam, 1953; Singsen *et al.*, 1955; Draper *et al.*, 1958). Johnson *et al.* (1958a) have shown that ingested BHT causes a reduction in the iodine value of perirenal fat in the rat, while Kummerow *et al.* (1949) established a further relationship between dietary antioxidants and the composition of depot fat when they showed that the synthetic antioxidant, butylated hydroxy anisole, like the tocopherols, is functional in the synthesis of oleic and saturated acids.

The above evidence, together with the results of this present study, suggests a tentative hypothesis that relatively large doses of BHT interfere with the normal metabolism of the fatty acids, this abnormal metabolism resulting in increased total serum cholesterol and phospholipid levels.

The tocopherols do not have the same action as BHT as, in the rat maintained on normal diets unsupplemented with cholesterol, no changes in blood cholesterol levels can be produced by hypervitaminosis E (Monnier *et al.*, 1941; Marx *et al.*, 1949; Aftergood *et al.*, 1957; Wissler *et al.*, 1958). BHT and three other synthetic antioxidants, when fed at a level of 0·025 p.c. in the diet, did not prevent the increase in plasma cholesterol and muscle lipid which occur before the onset of muscular dystrophy, induced in guinea-pigs by a vitamin E deficient diet. Supplements of α-tocopherol, fully protective against the occurrence of dystrophy, resulted in much lower levels in these lipid components (Shull *et al.*, 1958). In humans Gray and Loh (1958) have shown that ingestion of α-tocopherol produced increases in serum cholesterol, phospholipid, and the α_2 globulin fraction, but has no effect on the fatty acid content. BHT produces very similar effects in the rat, as it is probable that the α_2 globulin increases are analogous with the mucoprotein increases shown in the present study, especially as Bjornesjo (1955) has shown that the α_2 globulin fraction contains the greatest percentage of polysaccharide.

The increase in mucoprotein material upon ingestion of BHT suggests a biochemical connection between BHT and the synthesis or degradation of ground substance mucopolysaccharides in the body. Alternatively, such changes in mucoprotein metabolism may reflect ground substance changes, secondary to altered fatty acid metabolism.

Johnson and O'Halloran (1958b) have shown that there is an increase in the liver size of rats fed the NTD of BHT, which was not accompanied by any pathological or histopathological changes. They suggest that the liver is the site of detoxication of BHT, the end products of which include glucuronides and ethereal sulphates (Dacre, 1957; Deichmann *et al.*, 1955), the increase in liver size being a response on the part of this organ to the stresses placed upon it by the detoxication of an abnormal exogenous material. If the detoxication capacity of the liver should be inadequate to deal with the relatively large doses of ingested BHT, this substance or its metabolites may accumulate in the blood or tissues and be responsible for the effects reported in this study. The lack of an elevation in serum cholesterol and phospholipid at lower levels of dietary BHT, reported here, and those of Shull *et al.* (1958) using vitamin E deficient guinea-pigs, may be attributed to the relative ease of hepatic detoxication of BHT.

It is difficult to interpret the effects found in the paired feeding experiment, resulting from different dietary supplements of lard and restricted food intake on the serum cholesterol, phospholipid and hexosamine levels of the control animals. In this study there was no attempt to substitute isocalorifically the lard for some other component of the diet. Supplementation of the diet by lard, with a subsequent reduction in the percentage of other components, did not lead to any significant variations in the body weight or renal and hepatic histopathology of the animals (Johnson and O'Halloran, 1958b). The significant reduction of the serum cholesterol levels on supplementation of the diet by lard, a relatively saturated fat, is not in accord with observations on humans reported from many different laboratories, which have shown that in general the serum cholesterol level is inversely related to the iodine value of the dietary fat. Our work together with that of Bose and Subrahmanyan (1950) and Best *et al.* (1958), who could show no significant differences in the total serum cholesterol of rats fed different fats of varying iodine values, suggests that the rat differs markedly from man and other species in the response of serum cholesterol to variations in the amount and degree of unsaturation of the dietary fat.

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AN EFFICIENT, INEXPENSIVE DIALYSER

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SUMMARY.

A simple, efficient, small-scale, intermittent-rotation dialyser is described which can be constructed from readily available components at small cost.

INTRODUCTION.

If both are agitated, equilibrium between liquids inside and outside a dialysis membrane is achieved most rapidly. This may be attained by having stirrers in the liquids, but generally the surface of the dialysand must thereby be exposed to the atmosphere. If the osmotic pressure of the solute in the inner compartment is the greater a usually undesirable volume increase results. Rapid dialysis of volumes from a few ml. to 100 ml., with little increase in volume, can be achieved with the equipment described here, in which stirring is effected by rotation of the dialysis sac with intermittent stopping and reversal of the direction of rotation. A number of units may be employed if larger volumes have to be equilibrated.

METHOD.

The electrical components, all readily available at small cost, are a Venner 30 r.p.m. synchronous motor (type SM2S, 240 v. A.C.) with a 60 watt filament lamp in parallel and a Paynco No. 1 Flasher (100 watt max.), in series. The lamp provides sufficient current drain to operate the Flasher, an intermittent thermal switch used for advertising signs. The mechanical anti-reversing switch on the motor is disengaged, with consequent greater mixing effect, since then the motor can travel in either direction depending on the phase at starting.

The sac is prepared from a 12in. length of dialysis tubing (Arthur H. Thomas Co. No. 4465-A2, 1$\frac{1}{16}$in. flat width, was found most useful) which after soaking in water is tied with three knots 1in. apart at one end and these pulled tight. The section with the knots is tied around the neck of a large glass stopper which serves the double purpose of weighting the sac and stirring the liquid outside. The solution to be dialysed is poured into the sac, the top tied with two tight knots 1in. apart and looped over a wire connection of the boss on the motor shaft as illustrated in Fig. 1. Dialysis is performed either with regular changes of

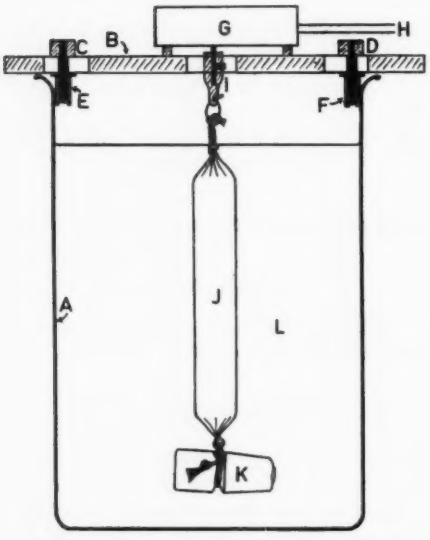

Fig. 1. *Dialysis equipment.* A, 3 l. beaker; B, wooden or plastic strip, 8in. x 2½in. x ⅜in.; C and D, sliding brass clips, running in slots in B; E and F, rubber tubes; G, synchronous motor; H, connections to Flasher and power supply; I, brass boss; J, dialysis sac; K, glass stopper; L, liquid in beaker.

Fig. 1. An efficient, inexpensive dialyser.

liquid in the beaker or with a continuous flow system, using a smaller outer container, with a side arm near the top to allow overflow.

The equipment will operate at 1° C., so that all or part may be placed in a refrigerator or cold room if desired. No trouble was encountered over two years' use of about once per week.